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STUDIES ON THE BIOLOGY OF THE NEUROACTIVE AMINES
A MORPHOLOGIC AND PHARMACOLOGIC APPROACH

HERBERT Y. MELTZER

1963

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STUDIES ON THE BIOLOGY OF THE NEUROACTIVE AMINES: A MORPHOLOGIC AND PHARMACOLOGIC APPROACH

Herbert Y. Meltzer

A thesis presented to the faculty of the
Yale University School of Medicine
in partial fulfillment of the
requirements for the degree of
Doctor of Medicine
1963

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The author gratefully acknowledges the indulgence and support of Drs. R.J. Barrnett, N.J. Giarman, D.X. Freedman, and J.P. Green who permitted this young investigator to explore his own ideas in their excellent laboratories. This thesis is dedicated to the memory of my late father, Mr. David Meltzer.

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PREFACE

The biology of the neuro-active amines has occupied a large portion of the imagination and energy of pharmacologists and physiologists investigating the vertebrate nervous system in the last decade. The basis for this concentration of interest are studies which implicate these amines in neurotransmission and, beyond that, through many derangements in their disposition from synthesis to destruction, in mental and nervous disorders. The investigations described and discussed in this thesis explore certain aspects of this class of compounds and the tissues which possess them.

In brief outline, with several simplifications, the life cycle of a neuro-active amine [e.g., 5-hydroxytryptamine (5-HT), histamine, norepinephrine (NE)] is: synthesis by decarboxylation of its precursor amino acid, storage in membrane-bound granules, release in response to various stimuli, action at the receptor site, and then either degredation by one or more enzymes or reabsorption by a neurosecretory cell for re-use.

The investigations in this work were concerned with: (i) the fine structure of the Dunn-Potter murine mast cell tumor grown in tissue culture and as ascites cells in the mouse peritoneal cavity; the origin of the amine granules of these cells and how they get the amines they contain; the mechanism of amine release by reserpine; the morphologic correlates of chlorpromazine action; the isolation of the amine-containing granules from the ascites mastocytoma cells; the absence of monoamine oxidase (MAO), an enzyme that has

PREFACE

The biology of the nerve-cells and fibres has occupied a large portion of the investigative and energy of physiologists and pathologists throughout the vertebrate nervous system in the last decade. The best, for this consideration of research are studies which implicate these cells in neurodegenerative and, beyond that, through many demyelinating or black degenerations, are problems in deterioration, in mental and nervous illnesses. The investigations described and discussed in this thesis concerns certain aspects of this class of degeneration and the disease which produces them.

In brief outline, with several simplifications, the pathogenesis of a pyro-acetic acidosis (6-4-, 2-dihydroxypyrimidine (6-HX), thiamine, nicotinamide (V.A)) is: acidosis by desaminolysis of the pyruvate molecule acid, absorbs in membrane-bound granules, which is necessary to vertebrate animals, which is the neurotoxic site, and then either degeneration by one or more enzymes or reabsorption by a neurosecretory cell for re-use.

The investigations in this work have concerned with:

(1) the fine structure of the hypo-thalamic nuclei and their cross in these animals and in addition which is the same particular cells; the study of the cells, particular of black cells and how they get the cellular body contents; the mechanism of cellular injury by neurotoxins; the morphologic correlates of electrocortical activity; the localisation of the amino-containing transfers from the acetic mechanisms which; the absence of neurotoxic cellular (TNC), in cases that has

been implicated in the degredation of several biogenic amines, and histaminase, the enzyme which oxidizes histamine, in these cells; (ii) the synthesis and study of a new class of inhibitors of MAO; and (iii) the relationship between the vitamin, thiamine, and MAO. The studies on the mastocytoma cells and the MAO studies will be presented separately for the sake of convenience and clarity.

been implicated in the development of several diseases, and discussed, the review will outline findings in these areas: (ii) the availability and study of a new class of inhibitors of [illegible]; and (iii) the relationship between the vitamin, biotin, and [illegible]. The results on the biochemistry and the [illegible] will be presented separately for the sake of convenience and clarity.

INTRODUCTION

The discoveries that histamine is found in the mast cells of a myriad of species,[1] that 5-hydroxytryptamine is found in the mast cells of the rat and mouse,[2,3] and that these amines are largely bound in granules[4] made this cell of great interest to the student of amine biology. It meant that the mast cell, along with the cells of the adrenal medulla and the blood platelets could serve as a model system for the amine biology of the vertebrate nervous system whose complexity makes a direct experimental approach extremely difficult. Thus, information about amine synthesis, destruction, binding, release, regulation, and the influence of hormones and drugs is much more easily obtained from the mast cell than from the vertebrate nervous system. And with the economy of mechanism that nature practices, it seems reasonable to hope that the information obtained from the mast cells would be of value in understanding many of the features of amines in the nervous system. This has indeed proven true. Investigators throughout the world have explored the mast cells with particular attention to their amines. It need not be stressed that the mast cell has great intrinsic interest in its own right.

[illegible]

The discoveries that interests is found in the rest cells of a variety of species,[1] that γ-hydroxybutyrate is found in the rest cells of the rat and mouse,[2,3] and that these salts are largely found in granules[4] made this cell of great interest to the student of cellular biology. It point that the rest cell, along with the cells of the adrenal medulla and the blood chromaffin could serve as a model system for the cellular biology of the vertebrate nervous system since considerably easier a direct experimental approach extremely difficult. Thus, information about amino synthesis, destruction, binding, release, regulation, and the influence of hormone and drugs is much more easily obtained from the rest cell than from the vertebrate nervous system. And with the concept of reticulum that rather practices, it seems reasonable to hope that the information obtained from the rest cells would be of value in understanding many of the features of amines in the nervous system. This has indeed proven true. Investigators throughout the world have explored the rest cells with particular attention to their amines. It need not be stressed that the rest cell has great intrinsic interest in its own right.

Outline of Experimental Investigations

The Yale Medical School Department of Pharmacology has been very active in the study of mast cells. The Dunn-Potter mouse mastocytoma[5] which has continued to produce histamine, 5-HT and heparin has been and is being studied by many people in that department. The author felt that these biochemical and pharmacologic studies could be greatly aided if the fine structure of these cells as seen at the level of resolution of the electron microscope could be established, because some of the studies undertaken have obtained a level of sophistication where function could not be divorced from structure. Indeed, even the simpler experiments could be more readily interpreted if the cells' morphology was known, for truly "one (electron microscopic) picture is worth a thousand words."

The Dunn-Potter mastocytoma can be grown as a solid tumor, as ascites cells in the mouse peritoneal cavity, or in tissue culture as a cell suspension. My first endeavour was to establish the fine structure of the ascites cell. It was then decided to use the tissue culture cells for studies using pharmacologic techniques and electron microscopy. The tissue culture cells are a pure population of mastocytoma cells whereas the ascites cells obtained from the mouse peritoneal cavity are really a mixture of mastocytoma cells, fibroblasts and leuokcytes. The tissue culture cells are more readily treated with drugs than the ascites cells and there is no interference from the host animal. The tissue culture cells were found to have a morphology significantly different from

Outline of Theoretical Investigations

The Yale Medical School Department of Pharmacology has been very active in the study of mast cells. The "non-binding mouse mastocytoma" which has contributed to studies initiated by E-M and Uyenoyama has been and is being studied by many people in that department. The author felt that these biochemical and pharmacologic studies could be greatly aided if the fine structure of these cells as seen at the level of resolution of the electron microscope could be established, because some of the studies undertaken have obtained a level of sophistication where function could not be divorced from structure. Indeed, even the simple experiments could be more readily interpreted if the cellular morphology was known. For truly "one (electron microscopic) picture is worth a thousand words."

The non-binding mastocytoma can be grown as a solid tumor, or mastocytoma cells in the mouse peritoneal cavity, or in tissue culture as a cell suspension. My first procedure was to establish the fine structure of the mastocytoma cell. It was then decided to use the tissue culture cells for studies using phase-contrast techniques and electron microscopy. The tissue culture cells are a pure population of mastocytoma cells whereas the mastocytoma cells obtained from the mouse peritoneal cavity are really a mixture of mastocytoma cells, fibroblasts and leucocytes. The tissue culture cells are more readily harvested with dense fixed the mastocytoma cells and there is no interference from the host animal. The tissue culture cells were found to have a morphologically significantly different from

the ascites cells and indeed, from any other mast cell previously described, but I decided to continue my studies with this cell form, fully realizing that it is a very atypical cell. The tissue culture cells have been investigated by Furano[6] who attempted to separate the amine-containing granules from the mitochondria of these cells by density gradient centrifugation. I sought to check his separation by electron microscopy but was unable to reproduce this work. Instead, an unseparated large granule fraction of these cells was observed in the electron microscope.

Several studies were undertaken with drugs which release mast cell 5-HT because it was felt that if the process could be visualized in the electron microscope valuable information about the mechanism of amine release would be forthcoming. It was hoped that such basic questions as whether amine release requires disruption of the cell and release of granules or whether it can occur without loss of cytoplasmic granules or other visible cytologic change could be answered. I hoped to obtain information about the mode of action of the drugs used, reserpine and chlorpromazine.

Of special interest were pictures of ascites mastocytoma cells which show what are thought to be early forms of the mast cell granules. These suggested a new theory for the origin of these granules.

Several studies of these mast cells performed by the author prior to the electron microscope studies will be reported briefly because they are of interest in interpreting some of the electron microscopic findings; (i) separation by density gradient centrifugation of subcellular components,

the nerve cells are defined, from the cytoplasmic cell particularly dangerous, but I decided to continue my studies with both cell lines, fully realizing that it is a very atypical cell. The giant nerve cells have been investigated by Hydén and attempted to separate the nucleo-containing portion from the protoplasmic of these cells by density gradient centrifugation. I hope to direct his separation by electron microscopy but was unable to remove this work. Instead, an unexpected large granule fraction of these cells was observed in the electron microscope.

Several studies were undertaken with dense which kills cell I-3 because it was felt that if the process could be visualized in the electron microscope valuable information about the secretion of nerve release would be forthcoming. It was hoped that such basic studies as whether nerve release results disruption of the cell and release of granules or whole, if can occur without loss of cytoplasmic granules or other viable cytoplasmic things could be observed. I hoped to obtain information about the mode of action of the drugs used, receptors and interpretation.

Of special interest were photures of nerve endoplasm cells which are thought to be early forms of the giant cell granules. These suggested a new theory for the origin of these granules.

Several studies of these giant cells performed by the author prior to the electron microscope showed it be reported initially because they are of interest in interpretation of the electron microscopic findings: (1) separation by density gradient centrifugation of nucleo-lipid components,

particularly the amine-containing granules and the mitochondria of the ascites mastocytoma cells; (ii) the demonstration of the absence or extremely low activity in the Dunn-Potter mastocytoma of MAO and histaminase.

particularly the audio-controlling processes and the audio-
observable of the analogic reproducing cells; (iii) the
demonstration of the absence of extremely low activity in
the human-hybrid analogy for the oral fixations.

The Mast Cell

The discovery of the mast cell is attributed to Paul Ehrlich who, in 1877, while investigating the staining of various tissues with aniline dyes, noted in the connective tissues of various species, granule-containing cells which had the property of staining more violet or redder than the rest of the cells (i.e. metachromasia).[7] He named these cells "Mastzellen" or well-fed cells because their cytoplasm was so densely crammed with granules. Ehrlich described the light microscope morphology, staining properties and distribution of the mast cells, but nothing of note elucidating their physiology or function was accomplished for seventy years. In 1937, Jorpes in Sweden noted that heparin, whose anticoagulant properties attracted great interest, stained metachromatically with toluidine blue.[8] Jorpes was then able to show that the most cell content of various tissues was proportional to its concentration of heparin.[1] Another big advance was the finding by Rocha e Silva that after anaphylactic shock both heparin and histamine were released from dog liver.[9] This led to a number of studies which firmly established the presence of histamine in mast cells.[1] Many other constituents of mast cells have since been found, including 5-hydroxytryptamine but the latter only in the mast cells of the mouse and rat.[2,3]

The Mast Cell

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Morphology of the Mast Cells

The morphology of the mast cell as seen with the light or phase microscope has been reviewed by Asboe-Nansen.[10] Mast cells may vary within wide limits in size and shape depending on many environmental as well as intrinsic factors. They may be flat, spherical, spindle-shaped, stellate or filiform. The rounded mast cells have a diameter of 8-15 microns, while the elongated ones may be twice as long. The nucleus is usually centrally located, about 4-6 microns in size and round or oval. Cells with two nuclei are occasionally seen but segmented nuclei have never been found.

According to Asboe-Hansen, mast cell granules are almost always globular-shaped, rarely oval. Elliptic or rod-shaped granules have been seen and attributed to pressure exerted by adjacent granules. Early investigators found the granules to be 0.2-0.4 microns in size, although one investigator, using freeze-dried mast cells from the rat mesentery found granules ranging in diameter from 0.3-1.0 microns.

Little else could be determined about the morphology of the mast cell, with the light microscope. The first electron micrographs of mast cells and normal mast cell granules were reported by Asboe-Hansen[11] and Köksal.[12] The techniques of electron microscopy were not very advanced at this time and little new information was obtained from these pictures. Köksal, however, did demonstrate a definite boundary to the mast cell granules.[12]

Morphology of the rest cells

The morphology of the rest cell as seen with the light or electron microscope has been reviewed by Jabon-Lemmon.[10] Rest cells may vary within wide limits in size and shape depending on many environmental as well as intrinsic factors. They may be flat, spherical, vacuolized, nucleated or filiform. The rounded rest cells have a diameter of 6-12 microns, while the elongated ones may be twice as long. The nucleus is usually centrally located, about 5-6 microns in size and round or oval. Cells with two nuclei are occasionally seen but segmented nuclei have never been found.

According to Jabon-Lemmon, rest cell granules are almost always globular-shaped, rarely oval. Filiform or rod-shaped granules have been seen and attributed to granules created by adjacent granules. Early investigators found the granules to be 0.2-0.4 microns in size, although one investigator, using freeze-dried rest cells from the red monkeys found granules smaller in diameter from 0.3-1.0 microns.

Little else could be determined about the morphology of the rest cell, with the light microscope. The first electron micrographs of rest cells are normal rest cell granules were measured by Jabon-Lemmon[11] and Kokes.[12] The techniques of electron microscopy were not very advanced at this time and little new information was obtained from these photos. Kokes, however, did demonstrate a definite boundary to the rest cell granules.[12]

The first electron micrographs of mast cells utilizing Palade's osmium tetroxide fixative[13] and thin-sectioning were obtained by Bloom et al. in Sweden.[14] The tissue studies was a dog mastocytoma which contained large amounts of heparin and histamine. The cells had the typical granular appearance of non-neoplastic mast cells. They were about 10 microns in size, round or oval in shape. The nucleus occupied from one-fourth to one-third of the cell volume and varied in shape from round to kidney configuration. The nucleus was surrounded by the usual nuclear membrane and had one or two nucleoli. The cytoplasm was filled with granules about 0.7 microns in size. Mitochondria with the usual fine structure were noted in mast cells for the first time. Some cells had large vacuoles in the cytoplasm. The cell surface had a great number of small filamentous cytoplasmic protrusions about 0.6 microns long and 0.08 microns wide.

Excellent electron micrographs of normal mouse mast cells were published by G.E. Rogers in 1956.[15] Phase microscopy and various histochemical techniques were used to demonstrate that certain granule-containing cells seen in the dorsal skin of three day-old mice were mast cells. The mast cell granules were quite electron dense and irregular in shape. Their size varied from 0.3 to 1.0 microns but were usually 0.5 microns wide. The number of granules per cell varied considerably but unlike most other mast cells that have been studied, did not occupy a large area of the cytoplasm of the cells. Each granule is usually located in a clear area in the cytoplasm which has no definite limiting membrane. The interior of the granule has

The first electron microscopic studies of yeast cells utilizing Kodak's osmium tetroxide fixative[20] and thin-sectioning were published by Glauert et al. in Sweden.[21] The fixative solution was a dry mixture which contained large amounts of hygroscopic sucrose. The cells had the typical granular appearance of non-nucleated bacterial cells. They were about 10 microns in size, round or oval in shape. The nucleus occupied from one-fourth to one-third of the cell volume and varied in shape from round to kidney configuration. The nucleus was surrounded by the usual nuclear membrane and had one or two nucleoli. The cytoplasm was filled with granules about 0.7 microns in size. Mitochondria with the usual tube structure were noted in each cell for the first time. Some cells had large vacuoles in the cytoplasm. The cell surface had a great number of small filamentous cytoplasmic protrusions about 0.5 microns long and 0.01 microns wide.

Excellent electron microscopic of budding cells were published by G.F. Cooper in 1960.[22] These microscopy and fixation biochemical techniques were used to demonstrate that certain mitotic-controlling cells seen in the dorsal skin of three day-old mice were mast cells. The mast cell granules were quite numerous there and irregular in shape. Their size varied from 0.3 to 1.0 microns but were usually 0.5 microns wide. The number of vacuoles per cell varied considerably but unlike most other mast cells that have been studied, did not occupy a large area of the cytoplasm of the cells. Mast granule is usually located in a clear area in the cytoplasm which has no definite limiting membrane. The interior of the granule has

the appearance of a network of filamentous elements. The cytoplasm contained an extensive well-organized agranular reticulum but a sparse endoplasmic reticulum. Mitochondria, both rounded and rod-shaped, with internal cristae were seen.

Other good electron micrographs of normal mast cells were published by Smith and Lewis in 1957.[16] They found that the mast cells from the spleen, spleen and liver capsules, mesentery, skin and peritoneal fluid of both the hamster and rat all had the same appearance. These cells were organized somewhat differently than the cells just described in that the granules were packed more closely together and the mitochondria appeared chiefly in groups in granule-free areas adjacent to the nucleus. However, elongated mitochondria were occasionally seen sandwiched in between the granules. An endoplasmic reticulum was also apparent in mast cell cytoplasm for the first time. The granules were round, oval, or irregularly shaped, 0.5-1.0 microns in size. High power views of them showed a reticular structure. They varied in osmophilia and thus some were much darker than others.

Hagen, Barrnett and Lee[17] published excellent electron micrographs of the Furth mouse mastocytoma[18] in 1958. The structure of these cells was again much different from any of those already described. The Furth mastocytoma possessed nuclei which tended to be irregularly shaped. The nucleus was bounded by double membrane which had frequent gaps or pores and was continuous with membranes of the agranular reticulum. The nuclei usually possessed one or more nucleoli.

the appearance of a number of filament-bearing elements. The cytoplasm contained an extensive well-organized system of vacuoles and a sparse endoplasmic reticulum. Chloroplasts, both bounded and well-aligned, with internal cristae were seen.

Other good electron micrographs of normal leaf cells were published by Dodd and Lewis in 1967.[10] They found that the leaf cells from the spleen, spleen and liver macrophages, neutrophils, skin and peripheral blood of both the controls and the cell had the same appearance. These cells contained organelles which differed from the cells just described in that the chloroplasts were packed more closely together and the mitochondria appeared cristae-rich in spite of granule-free areas adjacent to the nucleus. However, elongated mitochondria were occasionally seen scattered in between the granules. An endoplasmic reticulum was also apparent in each cell cytoplasm for the first time. The granules were round, oval, or irregularly shaped, 0.1-1.0 micron in size. Thin sector views of them showed a rectilinear lamellae. They varied in conspicuity and this seen from such factors they objects.

Hope, Lennert and Lee[11] published excellent electron micrographs of the Hodgkin cells Lymphocytes[12] in 1968. The appearance of these cells was from much different from any of those already described. The Hodgkin lymphocytes processed nuclei that tended to be irregularly shaped. The nucleus was bounded by double membrane which had frequent pores or pores and was continuous with membranes of the endoplasmic reticulum. The nucleus usually possessed one or more nucleoli.

The plasma membrane is very irregular. Small vesicles and cytoplasmic organelles are frequently seen adjacent to it. In many places a discrete membrane was not visible and cells were indistinctly demarcated from one another. "The cytoplasm is very complex. It contains a light homogenous matrix in which are embedded many cytoplasmic organelles, some of which are bizarre in form."[17] Many small irregular mitochondria with numerous cristae were scattered throughout the cytoplasm. There was a very extensive, complex agranular reticulum, varying in diameter from 200A° to 1,000A°, often with dense homogeneous material in the vesicles. The endoplasmic reticulum was seen much less often. The cells contained double-membraned vesicles as well as smaller vesicles and dense granules within one large vesicle. Large mastocytoma granules were frequently seen, usually scattered irregularly in the cytoplasm, but sometimes concentrated in one region of the cell. The granules, which varied in size and density, were occasionally seen in vacuole-like spaces.

Hibbs et al. recently published electron micrographs of normal human mast cells.[19] These cells do not look very complex and are akin to those in normal hamster and rat cells[16] and the dog mastocytoma.[14] The cells are round or oval in shape and contain numerous widely spaced granules which are about 0.75 microns in diameter. High power micrographs reveal that "each granule consists of lamellar groups, some in the form of scrolls, in close association with particulate material." The cells had single regular nuclei and an active cell membrane.

It is important to point out that all the mast cells just described, the only ones so far studied with the electron microscope, all were fixed in osmium tetroxide, dehydrated in alcohol and embedded in methacrylate, and all showed granules.

The author studied the fine structure of the Dunn-Potter mouse mastocytoma[5]. It is of interest to review the light microscope description of the tumor at its inception. It was noted about the solid tumor that: "No masses of intensely granulated cells closely resembling normal mast cells have ever been found in the transplanted tumors"[5]. The ascites tumor cells, stained with Wright's stain, were frequently found to have a ring or donut-shaped nucleus. The granules in a given cell were of the same size, but there was a great variation in the size of granules in different cells. The granules were not evenly distributed throughout the cell but tended to be clumped in one portion of it. The cytoplasm of less differentiated cells, with large round nuclei occuping most of the volume of the cell, usually contained small delicate granules. It is thus clear, that the Dunn-Potter mastocytoma had few granules even at its inception.

Distribution of Tissue Mast Cells

Mast cells are found in greatest abundance in the subcutaneous connective tissue, lung, mesentery, scrotum, uterus and thymus of mammals. The parenchyma of most organs have few mast cells but the fibrous tissue of their capsules and the connective tissue around small blood vessels is often

It is important to point out that all the mast cells just described, the all, were in the stained with the electron microscope, all were fixed in osmic tetroxide, dehydrated in alcohol and embedded in methacrylate, and all stained specially.

The author studied the fine structure of the non-myelin cause mastocytosis. It is of interest to review the light microscopic characteristics of the cells in the lymphoma. It was noted about the mast cells that: "In masses of tolerantly premalignant cells clearly proliferating normal mast cells have been found in the immunoblasted tissue". The mastocytic tumor cells, stained with Giemsa's stain, were frequently found to have a ring or donut-shaped nucleus. The presence in a given cell nucleus of this same size, but there was a great variation in the size of granules in different cells. The granules were not evenly distributed throughout the cell but tended to be clumped in one position of it. The cytoplasm of less differentiated cells, with large round nuclei occupied most of the volume of the cell, usually contained small delicate granules. It is thus clear, that the non-myelin mastocytosis had low granules even at the lymphoma.

Distribution of Mast Cells

Mast cells are found in greatest abundance in the sub-mucous connective tissue, lung, mesentery, nodes, spleen and thymus of mammals. The presence of mast cells in few mast cells but the fibrous tissue of their capsules and the connective tissue around small blood vessels is often

rich in them. The central nervous system has almost no mast cells but the connective tissue sheaths of peripheral nerves have many. Mast cells have been found in the tissues of almost all classes of animals in which they have been looked for.

The Pharmacology of the Mast Cell

Selected aspects of the pharmacology of mast cells, normal and neoplastic, will be reviewed. Particular attention will be paid to studies dealing with heparin, histamine and 5-HT, the sequestering of these substances within granules, and the effects of amine-releasing agents on these granules and the mast cells.

Mast Cell Heparin and Its Particulate Nature

Erik Jorpes, a Swedish biochemist, first proposed that the mast cells produced or contained heparin.[20] This hypothesis was based on the observation that there is a good proportionality between the number of mast cells and the amount of heparin in many tissues. Oliver *et al.* found that a dog mastocytoma contained fifty times as much heparin as normal dog liver.[21] Jorpes' observation that heparin, like the mast cell granules, is metachromatic, was the first evidence that heparin was contained within the mast cell granule.[22] Köksal later isolated mast cell granules from mouse connective tissues and showed them to contain a heparin-like anti-coagulant.[23] The synthesis of heparin by mast cells has recently been directly demonstrated.[24] Hagen,

with an axon. The central nervous system has glands so neat cells but the connective tissue elements of mesenchymal origin have many. These cells have been found in the tissues of almost all classes of animals in which they have been looked for.

The Neurochemistry of the Mast Cell

Selected aspects of the physiology of mast cells, normal and neoplastic, will be reviewed. Particular attention will be paid to studies dealing with heparin, histamine and 5-HT, the sequestering of these substances within granules, and the effects of radio-protecting agents on these granules and the mast cells.

Mast cell heparin and its particulate nature

Mark Jorpes, a Swedish biochemist, first proposed that the mast cells produced or contained heparin.[20] This hypothesis was based on the observation that there is a good morphotropicity between the number of mast cells and the amount of heparin in many tissues. Hilvert et al. found that a dog mastocytoma contained fifty times as much heparin as normal dog liver.[21] Jorpes' observation that heparin, like the mast cell granules, is metachromatic, was the first evidence that heparin was contained within the mast cell granule.[22] Kofoed later isolated mast cell granules from mouse connective tissues and assayed them to contain a heparin-like anti-coagulant.[23] The appearance of heparin by mast cells has recently been directly demonstrated.[24] Heparin,

Barrnett, and Lee demonstrated the presence of heparin the specific granules which were clearly distinct from mitochondria in the Furth mastocytoma.[17]

Mast Cell Histamine and Its Particulate Nature

The finding by Rocha e Silva *et al.*[9] that both histamine and heparin were liberated from the isolated dog liver during anaphylactic shock led J.F. Riley and others to investigate whether histamine too was produced and stored in the mast cell.[1] Utilizing the chemical histamine liberators discovered by MacIntosh and Paton,[25] Riley demonstrated that stilbamidine and d-tubocurarine caused disruption of mast cells.[26] However, these drugs quickly killed the animals and it was possible that the observations were due to agonal changes. Fawcett, using mast cells in rat mesentery, demonstrated that if the mesentery was depleted of mast cells by prior treatment with distilled water, intraperitoneal injections of compound 48/80 produced no histamine release, indicating that the mast cells were the source of histamine.[27] Quantitative studies suggested that the amount of histamine in the mast cells must have been extraordinarily high.[27]

Simultaneously, evidence was obtained through centrifugation studies that histamine is particulate bound rather than free in the cytoplasm.[4,28,29] The large granule fraction rich in histamine was found to contain many mast cell granules, identifiable by their metachromatic staining, and to contain few mitochondria indicating that the granules which contained

Unfortunately, we have misunderstood the presence of numerous specific granules which were clearly distinct from chondriosomes in the yeast sarcocysts.[17]

Yeast Cell Chondriosomes and the Cytoplasmic Granules

The finding by Hooke & Salvo et al.[9] that both chondriosomes and bacteria were liberated from the isolated sac fluid during anaphylaxis among the S.P. Kitty and others to investigate whether chondriosome loss was produced and observed in the yeast cell, initiated the classical chondriosome literature discovered by Hoskinson and Tyson.[20] Later demonstrated that antihumoral and a-humoral cytes caused disruption of yeast cells.[20] However, these drugs probably killed the cytoplasm and it was possible that the observations were due to normal changes. Therefore, using yeast cells in vitro cytochemistry, we demonstrated that if the mitochondrial was depleted of yeast cells by prior treatment with Triton in water, intracytoplasmic injections of commercial Lipo-20 succeeded in diabetic failure. Indicating that the yeast cells were the source of diabetes.[21] Quantitative studies suggested that the amount of liberation in the yeast cells just were been extraordinarily high.[21]

Similarly, evidence was obtained through conditioned studies that liberation in mitochondrial based yeast bites that was in the cytoplasm.[4,20,22] The large granule fraction rich in diabetes was found to contain many yeast cell granules, identifiable by their cytochromatic staining, and to contain low chondriosomes indicating that the granules which contained

histamine were not mitochondria.[4] Hagen et al. demonstrated that in the Furth mastocytoma, histamine was bound in granules which were morphologically and chemically distinct from mitochondria.[17]

Mast Cell 5-HT and Its Particulate Nature

The presence of 5-HT in rat mast cells was discovered by Benditt et al. in 1955. The only other species whose mast cells have definitely been found to possess 5-HT is the mouse.[3] The presence of 5-HT in the Dunn-Potter mouse mastocytoma and the skin lesions of two people with urticaria pigmentosa (which is characterized by an excess of mast cells[1]) was noted by Sjoerdsma et al.[30] 5-HT was not found in the mast cells of the human spleen.[31] Very recently, Enerbäck claimed he demonstrated 5-HT in mast cell granules in human carcinoid tumors.[32] Presumably, the mast cells took up excess 5-HT produced by enterochromaffin cells of the carcinoid tumor. The presence of 5-HT in the granules of the Furth mastocytoma was demonstrated by Hagen et al.[17]

The Release of Mast Cell Amines

It is not our aim to review the enormous amount of literature on the release of mast cell amines. Several aspects of this problem, however, are pertinent to our work: the release of amines as compared to the release of granules; the effect of release on cell morphology; the relationship between histamine release and 5-HT release; and the mechanism of the release process.

histamine were not antagonists.[6] Dreyer et al. demonstrated that in the Mouth mucoprotein, histamine was found in granules which were morphologically and chemically distinct from mitochondria.[17]

Mast Cell 5-HT and the Parkinsonian Tremor

The presence of 5-HT in mast cells was discovered by Rondelli et al. in 1953. The only other species where mast cells have definitely been found to possess 5-HT is the mouse.[3] The presence of 5-HT in the human-rodent mucoprotein and the skin lesions of the people with carcinoid diarrhoea (which is characterized by an excess of mast cells[1]) was noted by Sjoerdsma et al.[10] 5-HT was not found in the mast cells of the human spleen.[11] Very recently, Jarrbeck claimed to demonstrated 5-HT in mast cell granules in human carcinoid tumors.[12] Presumably, the mast cells took up excess 5-HT produced by enterochromaffin cells of the carcinoid tumor. The presence of 5-HT in the granules of the Mouth mucoprotein was demonstrated by Dreyer et al.[17]

The Release of Mast Cell Amines

It is not our aim to review the enormous amount of literature on the release of mast cell amines. Several aspects of this problem, however, are pertinent to our work: the release of amines as compared to the release of granules; the effect of release on cell morphology; the relationship between histamine release and 5-HT release; and the mechanism of the release process.

Mota et al.[33] demonstrated that Compound 48/80, a condensation produce of p-methoxyphenylethylmethylamine and formaldehyde which had been shown by Paton to liberate histamine,[34] produced a generalized disruption and release of granules from mast cells of rat mesentery. Parekh and Glick found that 48/80 did not cause any release of amines from intact mast cells short of absolute destruction of the cells.[35]

Similar findings were obtained by Bloom et al. who followed the morphological effects of more potent histamine liberators on the dog mastocytoma previously described. Compound 48/80 and stilbamidine produced "vacuolation and dissolution of mast cell granules as well as disintegration of the cell." The drug-induced vacuoles increased rapidly in size and number while the mast cell granules decreased in number. Some vacuoles contained granules and those near the periphery of the cell often ruptured, discharging their contents. The vacuoles were surrounded by a distinct wall which consisted of a double membrane.[36]

However, Fawcett showed that the extent of degranulation and histamine release in the rat mesentery was proportional to the amount of 48/80 present. He demonstrated that after injecting a Tyrode's solution containing 48/80 intraperitoneally into rats the mast cell granules were still present in the mesentery, but in the extracellular matrix surrounding each mast cell; histamine was now present in the Tyrode's solution in the peritoneum indicating that 48/80 had produced both

Cote et al.[23] demonstrated that Compound 48/80, a compound of unknown structure, morphologically similar and pharmacologically related to histamine, produced a prolonged diarrhoea and release of granules from rat cells of rat mesentery. Jacob and Spicer found that 48/80 did not cause any release of histamine from isolated rat cells short of absolute destruction of the cells.[24]

Similar findings were obtained by Bloom et al. who followed the morphological effects of mast cell degranulation on the dog mesenteric vascular bed. Compound 48/80 and antihistamine produced "vacuolation and disruption of mast cell granules as well as disintegration of the cell." The drug-induced vacuoles increased rapidly in size and number while the mast cell granules decreased in number. Some vacuoles contained granules and these were the periphery of the cell often ruptured, discharging their contents. The vacuoles were surrounded by a distinct wall which consisted of a cellic membrane.[25]

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degranulation and release of histamine from the cells.[27] It was not an all-or-none phenomenon in which the plasma membrane ruptured and all of the granules were released as part of a general destruction of the cell. The cells seemed to survive partial loss of their granules; they then underwent cell division and gradually regenerated their normal content of granules.

There is still a third correlation between histamine release and cell morphology. G.F. West has observed that if histamine liberators of low potency are used "histamine release can occur in a tissue without damage to its mast cell population." This is based upon light microscope studies.[37] Reserpine is one of the histamine liberators which West found released 5-HT maximally from rat skin but had little anatomic effect on mast cells as observed by light microscopy.[38] D.E. Smith reported that substantial release of histamine from mast cells can occur without degranulation in the presence of suitable concentrations of protamine sulfate and toluidine blue and considered this process closer to the physiologic release process than the massive degranulation produced by 48/80 or stilbamidine.[39]

It would seem that the effect of the releasing agents on cell morphology is a highly variable phenomena depending upon which agent and perhaps which type of mast cells are studied. It also appears that the amines may be released without releasing the granules and evidence will be cited shortly that the reverse is true as well.

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There is still a total correlation between histamine release and cell morphology. D.T. West has observed that if intradermal injections of low potency are used "histamine release can occur in a tissue without damage to its mast cell population." This is based upon light microscope studies.[28] Degeryne is one of the histamine liberators which most readily released D-MC mainly from rat with but little metabolic effect on mast cells as observed by light microscopy.[29] D.T. Smith reported that substantial release of histamine from mast cells can occur without degranulation in the presence of suitable concentrations of protamine sulfate and tolbutamide. One can consider this process closer to the physiologic release process from the reactive degranulation produced by D\80 or ritodrine.[30]

It would seem that the effect of the releasing agents on cell morphology is a highly variable phenomena depending upon which agent and perhaps which type of mast cells are studied. It also appears that the amines may be released without releasing the granules and evidence will be cited shortly that the reverse is true as well.

The release of 5-HT and histamine is often not parallel when mast cells which contain both these amines are exposed to releasing agents. Paratt and West investigated the effects of a series of releasing agents on the histamine and 5-HT content of the skin of rats: Polymyxin B produced extensive degranulation but little loss of 5-HT; morphine and Compound 48/80 produced equivalent depletion of both histamine and 5-HT along with widespread degranulation and disruption of mast cells; reserpine released more 5-HT than histamine and produced modest mast cell degranulation only after very large doses were administered.[40] Moran et al. confirmed that 48/80 releases equal percentages of 5-HT and histamine in rat mast cells.[41] A good deal of evidence was put forth by these authors in support of the idea that the release of 5-HT and histamine from the rat mast cells occurs by the same mechanism: identical time course of release of the two amines by 48/80, lack of selective release of either amine alone by any of the releasing agents tested (in contrast to Paratt and West's findings), comparable inhibition of release by chemical and thermal means and similar pH dependence.[41] Reserpine in concentrations of 150 micrograms/ml did not release either histamine or 5-HT.[41] Reserpine was found to be more active in releasing 5-HT than histamine from the brain, lungs and intestines of guinea pigs and rabbits.[42] Reserpine significantly decreased both 5-HT and histamine in the solid form of the Dunn-Potter mastocytoma.[31] It

The release of 5-HT and histamine in other tissues (lung, heart muscle, gut) is not certain but both these amines are suspected to initiating agents. Parker and Hunt investigated the effects of a number of releasing agents on the histamine and 5-HT content of the skin of rats: dodecylamin & produced extensive degranulation but little loss of 5-HT; morphine and compound 48/80 produced equivalent depletion of both histamine and 5-HT along with widespread degranulation and disruption of mast cells; reserpine released more 5-HT than histamine and produced modest mast cell degranulation only after very large doses were administered.[40] Lowe et al. confirmed that 48/80 releases equal proportion of 5-HT and histamine in rat mast cells.[41] A good deal of evidence was put forth by these authors in support of the idea that the release of 5-HT and histamine from the rat mast cells occurs by the same mechanism: identical time course of release of the two amines by 48/80, lack of selective release of either amine alone by any of the releasing agents tested (in contrast to Lewis and Lewis' findings), comparable inhibition of release by chemical and thermal means and similar pH dependence, marked in concentrations of 100 micrograms/ml and not present within histamine or 5-HT.[42] Reserpine was found to be more active in releasing 5-HT than histamine from the rabbit, human and intestines of guinea pigs and rabbits.[43] Reserpine significantly decreased both 5-HT and histamine in the mast cells of the Dom-Lefter mastocytoma.[37] It

releases 5-HT but not histamine from the same tumor grown as ascites cells.[43] But it releases both amines at extremely low concentrations from the same tumor cells grown in tissue culture.[44]

The question of the chemical basis of the action of the histamine liberators has been investigated extensively. One of the first suggestions and still widely held is that the histamine releasers displace histamine from its binding site, presumably heparin. This is based on the strong affinity of most histamine liberators for heparin.[45] Many studies showing that inhibitors of various enzymes block the release process have prompted the theory that the histamine liberators act by stimulating the action of an enzyme which brings about the release.[46-49] This has been persuasively disputed by van Arsdel and Bray who found no clear cut relationship between the enzyme inhibitors and blockade of 48/80's action.[50] Enzymatic processes have also been implicated in anaphylactic histamine release from tissues since several substances which inhibit enzyme action also inhibit histamine release and mast cell degranulation,[51] both of which have been shown to occur during antigen-antibody reactions.[46,52]

One of the important questions concerning the mechanism of release is whether the releasing agents act predominantly on the granules or on some other cell constituent. Certain evidence suggests it is a direct action on the granule: 48/80 and octylamine release histamine more rapidly from

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One of the important questions concerning the mechanism of release is whether the releasing agents act primarily on the granules or on some other cell constituent. Certain evidence suggests it is a direct action on the granule: [illegible] and compounds which release histamine more rapidly from

isolated granules from guinea pig lung than from lung minces;[53] similar findings have been noted with regard to the release of histamine from granules in dog liver.[54] McIntosh cites considerable evidence that the release of histamine involves rupture of the granule membrane: histamine release from isolated particles can be brought about by purely physical procedures which might be expected to disrupt a surface membrane such as suspension in hypotonic solutions, freezing and thawing, as well as surface-active agents such as saponin, bile salts, lysolecithin and the detergent Tween-20. McIntosh quotes Grossberg and Garcia y Arocho who suggested that "the final step, in any series of reactions leading to the release of histamine would be a change in the properties of the particle-cytoplasm interface, permitting the histamine to diffuse out. This change can be thought of most simply as a rupture or increase in the permeability of a membrane enclosing the particle."[55] McIntosh believes that an increase in permeability of the granules is brought about by the histamine liberators themselves even though these bases are only weakly surface-active in dilute solution.[55] As Paton has pointed out, one must consider the possibility that there is no one single mechanism of histamine release because there are so many types of histamine releasers: sensitizing compounds, toxin and venoms, proteolytic enzymes, surface-active agents, large molecules, histamine liberators, and monobasic compounds.[45]

Isolated granular fluorescence has been lost from most
[illegible]; [illegible] have been noted with [illegible]
to the release of [illegible] from granules in dog liver.[illegible]
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agents such as detergents, bile salts, lysolecithin and the
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the permeability of a membrane containing the granule."[illegible]
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types of [illegible] substances: [illegible] compounds, basic
and [illegible], [illegible] [illegible], surface-active [illegible], large
molecules, [illegible] [illegible], and [illegible] compounds.[illegible]

Other Constituents of Mast Cells

In addition to mucopolysaccharides such as heparin, and the amines, many other constituents have been found in mast cells: to name a few, albumen, glycoprotein, phospholipids, acid and alkaline phosphatase, lipase and cytochrome oxidase.[11] The enzymatic capacity of mast cells to synthesize amines from their precursor amino acids was reported by Hagen and Lee who demonstrated the presence of 5-hydroxytryptophane and histidine decarboxylase in the mast cell non-particulate cytoplasm.[56] The absence of histaminase and the presence of monoamine oxidase in the Furth mastocytoma has been reported by Hagen.[57]

Synthesis and Storage of Amines

Two of the leading unsolved questions of amine biology are the origin of the amine granules and how the amines get to the granules where they are stored. Cell fractionation studies have established that the amino acid decarboxylases which produce the amines from their precursors are present in the cell sap, that is, they are not bound to any structure as heavy as the endoplasmic or agranular reticulum.[58,59] The amines, then, are formed in the cell cytoplasm and must be conveyed to the granules or incorporated during formation of the granules. However, enzymes such as the mitochondrial monoamine oxidase [60] or non-particulate O-methyltransferase[61] or histaminase[62] could inactivate these amines after synthesis and before storage. The explanations that have been offered to this dilemma will be cited in the discussion of our findings

which permit us to postulate a new theory that explains the origin of the granules and the protection of the amines by a unified mechanism.

Function of the Mast Cells

The important question of the function of the mast cells is still far from settled, but we are in a far better position today to offer hypotheses than Ehrlich was in 1877. The presence of heparin, an anti-coagulant, and the location of tremendous numbers of mast cells along blood vessels, stimulated the idea that the cells are involved in endogenous anti-coagulation or in clearing lipids from blood, both well established functions of exogenous heparin.[20] The presence of the mucopolysaccharides heparin and hyaluronic acid in the mast cells suggested that the cells were important in laying down the ground substance of connective tissue. Recent work has tended to substantiate this thesis. The release of amines from mast cells has been shown by Riley to stimulate the mesenchymal cells to phagacytose and digest metachromatic material from nearby mast cells. The connective tissue cells then produce more mucopolysaccharide and new ground substance.[63] A mixture of heparin and histamine is required to produce local stimulation of mesenchymal activity: histamine alone is inadequate.[64] Wound healing is speeded by heparin.[65]

Histamine released from mast cells may also be important in the formation of bradykinin from one of the plasma globulins.[64] This peptide has already been shown to possess a potent vasodilator property, to increase capillary permeability, to cause

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Theories on the Lack Cells

The important question of the function of the lack cells is still far from settled, but we are in a far better position today to offer hypotheses than Elwinslokwa in 1877. The presence of lacunae, or anti-nucleus, and the lacunae of transmission ducts of lack cells along blood vessels, which-lead the fact that the cells are involved in enzymatic dematerialization or in storing light from blood, both well established facts of biochemistry of organism impact.[20] The presence of the mucopolysaccharides heparin and hyaluronic acid in the lack cells suggested that the cells were important in laying down the ground substance of connective tissue. Recent work has tended to substantiate this theory. The release of matrix from lack cells has been shown to play to obtained the mucopolysaccharides to glycoproteins and direct histochemistry indeed of free lack cells. The connective tissue cells then produce more mucopolysaccharides and fur-nished substance.[23] A studies of heparin and interacting is required to produce local alkalization of mucopolysaccharides activity: histamine alone is insufficient.[24] Lack heparin is opposed by heparin.[25]

Histamine released from lack cells may also be important in the formation of hyaluronic from one of the plasma globulins.[26] This peptide has already been shown to possess a potent vasodi-lator property, to increase capillary permeability, to cause

the accumulation of leukocytes, and to produce pain.[64]

It is well established that both histamine and 5-HT increase capillary permeability. This has led to the suggestion that after injury the mast cell releases histamine and (in the rat and mouse) 5-HT and that these are among the factors involved in the hyperemia and increased vascular permeability which occurs as a response to injury.[66] Draper and Smith showed that in rats whose peritoneum had been depleted of mast cells by an intraparitoneal injection of distilled water, there was an increased extravasation from capillaries of the peritoneum following a passively induced antigen-antibody reaction. This led them to postulate that mast cells may contribute to the initiation of the inflammation which follows antigen-antibody reactions.[67]

Reserpine

Reserpine and chlorpromazine are the drugs whose effects on mast cells morphology was investigated with the electron microscope by the author.

The similarity in the effects of reserpine and 5-HT on mice were the initial clues that the effects of reserpine were due to amine release.[68] It was later shown that reserpine increased the excretion of a metabolite of 5-HT, 5-hydroxyindoleacetic acid.[68] Direct measurement of 5-HT levels after reserpine treatment demonstrated that it lowered the 5-HT content of intestine, brain, platelets of many species and rat mast cells.[68] Reserpine also releases catecholamines from tissues including the brain, heart,

the accumulation of lactobacilli, and to produce pyuria.[64]

It is well established that both histamine and 5-hydroxytryptamine cause increase capillary permeability. This has led to the suggestion that either injury the mast cell releases histamine and/or the 5-HT and mast cell release 5-HT and that both are found in the factors involved in the inflammatory and increased vascular permeability which occurs as a response to injury.[65] Thus one might argue that in this study pentobarbital had been injected or mast cells by an intraperitoneal injection of stabilized water, there was an increased extravasation due capillaries of the peritoneum following a heavily induced antigen-antibody reaction. This led them to speculate that mast cells may contribute to the initiation of the inflammatory which follows antigen-antibody reactions.[66]

Conclusion

Reserpine and chlorpromazine on the charge alone effects on mast cells morphology was investigated with the electron microscope by the author.

The similarity in the effects of reserpine and 5-HT on mast cells the initial clues that the effects of reserpine were due to some release.[67] It was later shown that reserpine increased the excretion of a metabolite of 5-HT, 5-hydroxyindoleacetic acid.[68] Direct measurements of 5-HT levels after reserpine treatment demonstrated that it lowered the 5-HT content of rabbits, dogs, guinea pigs, many species and rat mast cells.[69] Reserpine also releases catecholamines from stores including the brain, heart,

ganglia, arteries and blood.[68] It lowers histamine levels in rabbit blood but not in other rabbit tissues.[68] Reserpine is more effective in releasing 5-HT than histamine from the mast cells of rat skin[38] and the Dunn-Potter mastocytoma.[39] Studies with the latter tumor grown as free cells in tissue culture by Giarman et al. showed that reserpine at 10^{-9}M produced approximately 50% release of 5-HT and 10^{-7}M, 100% release.[44]

Since these amines are particulate bound in many tissues, the effect (s) of reserpine on these particles in vitro in an isolated state is of great interest. Various histamine liberators such as 1,10-diaminododecane, propamidine and Compound 48/80 have been shown to release histamine from isolated mast cell granules. However, the release process differed from that observed in vivo in that the time course of the reaction was a 1st order reaction, proportionate to the concentration of both granules and releasing agent. The explosive release seen in intact tissues did not occur.[34,70] Walaszek and Abood found that reserpine released 5-HT from an isolated large granule fraction of rat brain.[71] Whittaker, however, found that reserpine did not release 5-HT from isolated brain particulate nor did it suppress uptake of 5-HT by these particles. However, particles depleted of 5-HT could be obtained from animals treated with reserpine.[72] Von Euler found that the effects of reserpine on NE-containing particulated isolated from splenic nerves varied with the concentration of reserpine at concentrations of 1-10 micrograms/ml it prevented the spontaneous release of

NE but in higher doses (100 micrograms/ml) it enhanced release.[73,74] These studies indicate that reserpine can exert a direct effect on the amine-containing granules *in vitro*. But this does not necessarily mean that the same is true *in vivo* or that this is the sole mechanism operating *in vivo*.

Other studies indicated that *in vivo* reserpine can either release the amine granules from the cells which contain them or disrupt them intracellularly. Marks *et al*. did a quantitative count of the 5-HT containing granules of enterochrohmaffin cells with the light microscope before and after reserpine treatment *in vivo* and found that the extent of degranulation at eight and sixteen hours paralled the changes in 5-HT content.[75] Burn *et al* found that reserpine decreased the number of chromaffin cells(?complete degranulation) and decreases the number of granules in the remaining cells in the skin of cat's tail and the nictitating membrane.[76] Electron microscopic studies were carried out by De Robertis and his colleagues using the rat pineal gland which has been shown to contain high concentrations of catechol amines,[77] 5-HT[77] and N-acetyl-5-methoxytryptamine.[78] De Robertis found that the pinealocytes of rats have "club-shaped perivascular expansions" containing vesicles of various types, some of which had dense granules that might contain the osmophilic amines known to be present in the pineal. Reserpine was observed to produce almost complete disappearance of the heterogeneous vesicles containing dense granules between two and forty-eight hours after a single injection. The time course of regeneration of the granules

over an eight day period corresponds strikingly with the recovery of 5-HT and NE in the brain of rabbits after treatment with reserpine.

Chlorpromazine

The other drug whose effects on mast cells were studied, was chlorpromazine, a very effective tranquilizer. Two factors prompted the choice of this particular pyschotropic agent. First, recent work in these laboratories demonstrated that chlorpromazine, like reserpine, releases 5-HT and histamine from the Dunn-Potter mastocytoma.[80] Chlorpromazine also releases 5-HT from platelets, by a mechanism which appears to be different than that of reserpine.[81] Secondly, although most of the biochemical work with chlorpromazine has been concerned with its effects on various enzyme systems (to be reviewed later), a current theory of chlorpromazine's mode of action considers its effects on various membranes with the cell,[82] particularly those of the mitochondria,[83] as being of central importance. Spirtes and Guth have shown that chlorpromazine inhibits the water imbibition of isolated mitochondria produced by thyroxine, detergents and phosphate.[83,84] It inhibits the uptake of 5-HT by platelets,[85] the uptake of adrenaline *in vivo* from the blood into tissues,[86] the *in vitro* release of acetylcholine from isolated storage organelles,[87] the metrazol-enhanced passage of dyes into the brain,[88] the absorption of drugs from subcutaneous tissues,[89] the intestinal absorption of lipids and sugars,[90] and the release of brain amines by reserpine.[91] All these

over an eight day period exposure took place, the
recovery of [illegible] in living tissue of rabbits after exposure
[illegible] with [illegible].

Conclusions

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and the [illegible] of [illegible] [illegible] by [illegible].[42] All these

effects might be explained by the accumulation of chlorpromazine which is lipid soluble in the lipoprotein of the cell membranes where it might alter permeability either directly or through various enzymatic processes.[92] The electron microscope might be of great value in demonstrating a morphologic effect of chlorpromazine on cell membranes if such exists. Roizin et al. believe that they have demonstrated with the electron microscope that chlorpromazine increased the osmophilia and reduced the size of mitochondria of nerve cells and neuroglia in biopsy specimens of rat brain and monkey cerebral cortex.[93]

The Dunn-Potter Mouse Mastocytoma

The spontaneous appearance of mast cell neoplasms in mice and other animals has been noted many times.[94] However, only two of these have been successfully transplanted and found to maintain their mast cell characteristics in successive generations.[5,17,18,31] The present investigation is concerned with one of these tumors, the P-815 mouse mastocytoma reported by Dunn and Potter in 1957.[5] A good deal of information about this tumor has already been reviewed in this Introduction. This tumor arose in a mouse of DBA f/2 strain whose skin had been repeatedly treated with methylcholanthrene. The morphology of the original tumor at the light microscope level has already been described (p.12). Sjoerdsma et al. established that this tumor produced histamine and 5-HT.[31] An ascites line of these cells was

efforts might be explained by the accumulation of photoproducts which is likely because in the chromosomes of the cell nucleus where it is not other permeability, either directly or through various enzymatic processes.[52] The electron microscope which has of great value in determining a morphologic effect of chlorpromazine on cell membranes if such exists. Bolero et al. believe that they have demonstrated with the electron microscope that chlorpromazine impaired the permeability and reduced the size of mitochondria of Ehrlich cell and mammalian liver mitochondria of rat liver and noted dilated cisterna.[53]

The Dunn-Hobbs cell transformation

The erythrocyte appears to be the cell most susceptible to this and other cellular lysis has been noted many times.[54] However, only two of these have been successfully transplanted and found to maintain their cell characteristics in successive generations.[Dunn, Hobbs][31] The present investigation is concerned with one of these tumors, the Z-K[illegible] round hepatoma reported by Dunn and Hobbs in 1947.[illegible] A good deal of information about this tumor has already been reviewed in this introduction. This tumor arose in a mouse of DBA type which was fed hay, regularly, treated with methylcholanthrene. The morphology of the regular tumor is the light microscope level has already been described (Hobbs). Bjorkman et al. established that this tumor produced histamine and [illegible][illegible] in another line of these cells was

established by intraperitoneal injection of a tumor brei.[5] Schindler et al. developed a medium that permits growth of the cells in vitro; the medium contains high concentrations of folic acid and undialyszed horse serum in addition to the usual constituents for tissue culture. Pure cell lines were established by isolating single cells and allowing them to multiply on feeder layers of chick embryo fibroblasts. A cell line called the T-Line which produced much larger amounts of 5-HT and histamine than the original line was established. From the T-Line two other lines were established by two successive cloning procedures, the X-1 and X-2 lines, each descendent from a single cell and thus each genetically pure.[95] The X-1 is a near-teraploid line, the X-2, a near-diploid. Both these clones produce even more 5-HT and histamine than the T-line. The cells grown in tissue culture contain more amines than those grown in mice.[96] With the solid mastocytoma, 70-85% of the amines and heparin was found in the fractions containing unbroken cells, nuclei, mitochondria and microsomes. These fractions were found to adsorb 40% of added 5-HT, histamine and heparin following addition to the homogenates of quantities of these compounds of comparable magnitude to those originally present.[96] This points up the well known difficulty of interpreting data from homogenization and centrifugation studies. Not only does the homogenization release granular bound material, the centrifugation procedures can cause factitious distribution of the released material by selective adsorption.

[illegible]

METHODS

Growth of ascitic cells

The ascites line of the Dunn-Potter mastocytoma was carried by innoculating approximately 1 X 10^4 mastocytoma cells in isotonic saline intraperitoneally into DBA mice. After a period of seven days, the animals were killed by cervical dislocation, the peritoneum was exposed, and the cells were washed out of the peritoneal cavity with isotonic saline. Aliquots were taken for 5-HT assays. The cells were then centrifuged into a pellet and prepared for electron microscopy.

Growth of tissue culture cells

The same cell line that was carried in the ascitic fluid was grown as a cell suspension *in vitro* in a special tissue culture medium particularly rich in folic acid.[5] The cells were not allowed to reach a concentration greater than 4 x 10^5/ml. When sufficient cells were present, they were harvested by centrifugation. Aliquots were taken for 5-HT assay. The number of cells present was determined by the use of a Coulter counter. The cells were then centrifuged into a pellet and prepared for electron microscopy.

Reserpine Treatment

The tissue culture cells were incubated at 37°C for 24 hours with reserpine at concentrations of 10^{-7}M or 10^{-9}M. Controls containing no reserpine were studied simultaneously. Aliquots were taken for 5-HT assay and for cell counts.

The reserpine-treated cells and the controls were then prepared for electron microscopy.

Chlorpromazine Treatment

The tissue culture cells were incubated for 12 hours at 37°C with chlorpromazine at a concentration of 1×10^{5}M. Controls containing no chlorpromazine were studied simultaneously. Aliquots were taken for 5-HT assay and for cell counts. The chlorpromazine-treated cells and the controls were then prepared for electron microscopy.

Light and Phase Microscopy

The mast cells were observed with the light and phase microscope in the unfixed condition, after fixation with 1% osmium tetroxide, and after staining with 0.1% Azure A in 30% alcohol (for metachromasia).

Electron Microscopy

Review of the literature on the electron microscopy of cultured or ascitic cells revealed that the best results have been obtained when the cells were centrifuged into a pellet and treated as a small tissue mass rather than by working with a loose suspension of cells.[16,97,98] Preliminary studies confirmed this about the mastocytoma cells. Therefore, for all the electron microscopic work, the ascitic cells and tissue culture cells were centrifuged into a pellet from a 0.30M sucrose media and the pellet was then gently disrupted to produce many small fragments. These were then fixed, dehydrated, and embedded according to standard techniques.[99]

The mercaptan-treated cells and the controls were then compared for electron microscopy.

Chlorpromazine Treatment

The tissue culture cells were incubated for 24 hours at 37°C with chlorpromazine at a concentration of 1 x 10^{-5}. Control cultures containing no chlorpromazine were studied simultaneously. Aliquots were taken for [illegible] assay and for cell counts. The chlorpromazine-treated cells and the controls were then prepared for electron microscopy.

Light and Phase Microscopy

The dead cells were observed with the light and phase microscopes in the stained condition, after fixing with 1% osmium tetroxide, and after staining with 0.1% trypan blue 95% alcohol (for phase microscopy).

Electron Microscopy

During or the litterature of the electron microscopy of cultured or ascitic cells revealed that the best results have been obtained when the cells were centrifuged into a pellet and treated as a small tissue mass rather than by working with a loose suspension of cells.[16,27,28] Preliminary studies confirmed this about the mercaptan cells. Therefore, for all the electron microscopic work, the ascitic cells and tissue culture cells were centrifuged into a pellet from a 0.9% sucrose media and the pellet was then gently dispersed to produce many small fragments. These were then fixed, dehydrated, and embedded according to standard techniques.[29]

which briefly are: fixation for 60 minutes in 1% osmium tetroxide buffered with veronal acetate to pH7.3, dehydration by a series of graded alcohols to absolute alcohol, embedding in prepolymerized methacrylate (80% methyl, 20% butyl) after saturating the tissue with this unpolymerized mixture, and polymerizing overnight at 60°C using dibenzoyl peroxide as catalyst. The condition of the cells was checked during these procedures by light and phase microscopy. In several instances, the cells were embedded in Epon according to the method of Luft.[100] Thin sections were cut on a Porter-Blum microtome and mounted on grids containing a collodion film. The sections were then observed with an Akashi electron microscope.

Isolation of Subcellular Components From the Ascites Cells

The aim of this work was to isolate a purified preparation of mast cell granules uncontaminated by mitochondria. Such a preparation would be of great value in characterizing the granules.

The ascites cells were chosen because they represented a source of mast cells easily available and relatively uncontaminated by other cell types. The first problem was to rupture the cell membrane and release the cytoplasmic inclusions. This proved to be quite a difficult problem. Sonic oscillation, high speed oscillation with small glass beads and freezing and thawing proved inferior to homogenization with the Potter-Eveljhem homogenizer. 10-15 minutes of homogenization at high speeds at 4°C ruptured over 60% of a 10% suspension of mast cells in 0.3M sucrose. Undoubtedly some constituents

which partially cover filaments for the formation of random textured silicon with vertical cracks to pH.7, degradation by a factor of greater elements or absolute [illegible], embedding a hydrogenated amorphous film across, or [illegible]) after subjecting the films with this [illegible] mixture, and polycrystalline overlayer at 400° using diffusion provide an indicator. The condition of the cells was checked during these procedures by light and dark electrons. In several instances, the cells were subjected to [illegible] on the region of [illegible].[100] Dark evaluations were out on a [illegible]-like electrodes and mounted on grids containing a collector film. The results were then compared with [illegible] electrons.

Fabrication of Photovoltaic Cells from the Amorphous Phase

The aim of this work was to fabricate a practical [illegible] of [illegible] cell grades unsatisfactory in photovoltaic. Such a procedure would be of great [illegible] in electrochemistry the [illegible].

The amorphous cells were chosen because they represented a source of [illegible] cells easily available and relatively [illegible] by other cell types. The first problem was to [illegible] the cell [illegible] and relieve the [illegible] electrons. This proved to be quite a difficult problem. [illegible] conditions, high speed [illegible] with small [illegible] and freezing and thawing proved [illegible] to [illegible] with the [illegible]. 10-14% [illegible] of [illegible] at high speeds at 4°C [illegible] of a 100 [illegible] of [illegible] cells in 0.25% [illegible]. [illegible] were [illegible]

originally present in the granules were released by this prolonged homogenization.

The presence of 5-HT, histamine and heparin were used as markers for the amine granules. Succinoxidase was used as a marker for the mitochondria.

Differential centrifugation of the suspension of the homogenized mast cells at various speeds and lengths of time was inadequate to separate the amine granules from the mitochondria.

Therefore, the technique of density gradient centrifugation was tried.[104-106] With this technique, a solution containing the particles to be separated is layered on top of a column of sucrose solutions of increasing density in a plastic test tube which can be centrifuged in a "swing-out" rotor at high speeds. Particles with different sedimentation rates are separated into discrete zones which can then be separated from one another by a variety of techniques.

A great deal of work was necessary before the optimal conditions for separating the granules and mitochondria of the ascites mastocytoma cells were found. Even then not all the mitochondria could be separated from the granules indicating that some mitochondria have the same sedimentation rate as the granules. The following procedure gave the most successful separation of granules and mitochondria.

Mice containing Dunn-Potter X-1-C ascites cells were given S^{35}- sulfate sixteen hours prior to isolation of the cells. The cells were washed several times with isotonic saline and then a 10% suspension in 0.3M sucrose was

homogenized in a Potter-Eveljhem apparatus at 4°C until the majority of cells were broken. Unbroken cells, nuclei and debria were removed from the homogenate by centrifugation at 700g for 10 minutes. The supernatant was decanted and a large granule fraction was separated from it by centrifuging at 20,000g for 30 minutes. The resulting precipitate was suspended in 5 cc's 0.3M sucrose, homogenized gently with the Potter Eveljhem homogenizer and layered gently over the sucrose density gradient.

This density gradient consisted of 10cc. of 1.7M sucrose, 5 cc of 1.2M sucrose and 5.cc of 0.8M sucrose each layered carefully on top of one another so as to minimize mixing. The large granule fraction in 0.3M sucrose was layered over the 0.8M sucrose and the fluid column in a clear plastic test tube was centrifuged for 60 minutes at 25,000 rpm in a Spinco -SW 25 rotor.

The centrifugation procedure produces several bands of particulates at varying densities. A Spinco tube-cutting device was used to cut the tube so as to be able to separate each band cleanly. Aliquots of these fractions were then used for analytical purposes. On one occasion one fraction was subjected to density gradient centrifugation on a second gradient in an attempt to obtain a better separation.

Isolation of Subcellular Components from the Tissue Culture Cells

The same techniques that were just described were later used by Furano to separate these components of the cultured mastocytoma cells.[6] We attempted to reproduce this work so as to obtain isolated granules for electron microscopy but

were unsuccessful. All the cytoplasmic material that was layered on top of the gradient traversed the entire gradient and was found in pellet form on the bottom of the test tube. This pellet was used for electron microscopy, nevertheless.

Determination of 5-HT

5-HT was determined spectrofluorometrically by the method of Udenfriend et al.[101] 5-HT was extracted into aqueous butanol from an alkaline-borate buffer solution saturated with sodium chloride. It was then extracted into dilute hydrochloric acid in the presence of heptane which is miscible with butanol and drives the 5-HT into the hydrochloric acid. The quantity of 5-HT present in the hydrochloric acid is then determined by the fluorescence in an Amino spectro-fluorometer, when activated at 295 millimicrons and read at 550 millimicrons in a concentrated hydrochloric acid solution. A series of standards was carried through the same extraction procedure to quantify the fluorescence.

Determination of Histamine

Histamine was determined spectrofluorometrically by the method of Shore et al. A 0.IN hydrochloric acid extract of the mast cells was made alkaline with sodium hydroxide and saturated with sodium chloride. It was then extracted into n-butanol. This was then shaken with heptane and hydrochloric acid to transfer histamine to the hydrochloric acid. Histamine was then coupled with orthophthaldialdehyde in an alkaline medium and the resulting fluorophore is measured at 450 millimicrons after activation at 360 millimicrons. Appropriate standards and controls were run through the same procedure.

were unsuccessful. All the chromatogram samples that was
imposed on top of the chromatogram demonstrated the marine penicillin
was found in polar fluid on the surface of the chromatogram.
This polar was used for the further microscopic observations.

Determination of L-Ser

L-Ser was identified chromatographically by the
method of Chatterjee et al.[illegible] L-Ser was extracted from
aqueous hydrolysate from an alcohol-phenol mixture and
separated with sodium chloride. It was then determined using
ninhydrin-cadmium acid in the presence of sodium from its
acetate buffer and finally the L-Ser from the phosphoric
acid. The quantity of L-Ser present in the phosphoric
acid was determined by the fluorescence in an
amino spectro-fluorometer, using excitation at 320 milliamicrons
and zero at 390 milliamicrons in a concentrated hydrochloric
acid solution. A series of standards was prepared simply as
were unknowns in quantity the fluorescence.

Determination of Tryptophan

Tryptophan was determined quantitatively by the
method of Chang et al. A 0.2% hydrochloric acid solution of the
crude cells was made alkaline with sodium hydroxide and adjusted
with phosphoric acid. The pH was then measured into a volume.
This was then shaken with hexane and hydrochloric acid to
remove lipids to the hydrochloric acid. Ninhydrin and
then complex with cadmium/hydrochloric in an alkaline medium
and the resulting fluorescence is measured at 320 milliamicrons
after excitation at 390 milliamicrons. Tryptophan standards
and controls were run through the same procedure.

Determination of Heparin

Heparin was determined by using radioactive S^{35}-sulfate as a tag.[96] These mast cells can incorporate S^{35} -sulfate into heparin. The amount incorporated was related directly to the amount of heparin in the cells.[96] The radioactive sulfate was injected intraperitoneally into rats one day before sacrificing the animals. Heparin was extracted from the sample to be analyzed by placing the sample together with an excess of pancreatin in a dialysis bag. It was then dialyzed against tris buffer pH 8.4 for twenty four hours and against running tap water for twelve hours. The mixture was centrifuged and the radioactivity of the supernatant was used as a measure of the amount of heparin present. The radioactivity of an aliquot of this material was measured in a Tri Carb liquid scintillation spectrometer by the usual means.

Determination of Succinoxidase

Succinoxidase was determined by the method of Slater.[103] The oxidation of succinate was coupled to the reduction of potassium ferricyanate which was quantitated spectrometrically. Sufficient potassium cyanide was added to inhibit cytochrome oxidase. Succinoxidase was used as a marker for the mitochondria.

Attempts to Demonstrate Monoamine Oxidase Activity in the Dunn-Potter Mastocytoma.

1. Techniques of Cotzias and Greenough[107]

The tissue to be assayed for MAO activity is incubated with tyramine or 5-HT as a substrate in a phosphate buffer at pH 7.4 at 37°C for 30 minutes. The reaction is stopped

with sodium carbonate. The ammonia liberated by the deamination of tyramine is transferred to a dilute hydrochloric acid solution by bubbling air through a test tube containing the reaction mixture connected by tubing to the hydrochloric acid solution. The ammonia in the hydrochloric acid solution is then determined with Nessler's reagent.[108] Appropriate controls without substrate or with heat inactivated enzyme are always used. Known monoamine oxidase inhibitors such as iproniazid[109] were also used to check if the ammonia liberated was due to monoamine oxidase activity.

The solid mastocytoma tumor as well as the ascites cells were used as the enzyme source. The cells were disrupted by homogenization, sonication, lysozyme, or freezing and thawing in order to liberate the enzyme. Mitochondria isolated from the equivalent of a gram of tissue were used in some experiments.

2. Technique of Weissbach et al:[110]

Because the previous methods failed to demonstrate any monoamine oxidase activity, a more sensitive assay technique was tried. This involved measuring the disappearance of kynuramine, a substrate of monoamine oxidase, spectrophotometrically. Purified preparations of mastocytoma mitochondria from up to one gram of tissue was used as the tissue source.

Attempts to Demonstrate Diamine Oxidase Activity in the Dunn-Potter Mastocytoma

Diamine oxidase, the enzyme which oxidizes histamine in mammalian tissues,[112] was sought by adapting the techniques of Cotzias and Greenough previously described, using histamine as a substrate.[107]

with boiling nitrobenzene. The product liberated by the decarboxylation of gramicidin is precipitated as a yellow gummy material and purified by dissolving the precipitate in ether, centrifuging the resulting mixture recovered by adding to the hydrochloric acid solution. The acidity of the hydrochloric acid solution is then determined with bromphenol reagent.[]

Hydrophilic colloids without substrates or with such substrates are stained red. Hence bromphenol colloids (colloidal emulsions) such as hydrosols[] were also used to ensure if the acidity liberated was due to proteolytic enzyme activity.

The acid substrates used as well as the enzyme solution were based on the enzyme source. The cells were disrupted by homogenization, sonication, lysozyme, or freezing and thawing in order to liberate the enzyme. Chloroform toluene from the equivalent of a pint of culture were used to some experiments.

3. Technique of assessment of [illegible]:

Testing the proteolytic activity failed to demonstrate the secondary cellular activity, a non-sensitive assay technique was tried. This involved measuring the disappearance of hydrolysis, a substrate of non-zero colloids, qualitatively and/or quantitatively. Further considerations of methodology differences from up to now used of fluid was used as the bacteria source.

Attempts to increase the enzyme activity in the culture fluid and medium

Attempts continue, the enzyme still exhibits [illegible] activity.[] [illegible] by adding the tentative of cellular and [illegible] [illegible], [illegible] as a substrate.[]

RESULTS AND DISCUSSION

The Ascitic Dunn-Potter X-1-C Mastocytoma Cell

The ascitic form of the Dunn-Potter mastocytoma was the first form of this tumor the author studied with the electron microscope. The cells are either round or elongated and vary from ten to twenty microns in size (Figs 1-3). The nuclear-cytoplasmic ratio is greater in the rounded cells than the elongated cells; the nuclei usually have one or two nucleoli and are surrounded by a double membrane.

The numerous long, thin pseudopodia of the plasma membrane, which in some places are forming vacuoles, indicates a high degree of surface activity in the elongated cells. The plasma membrane is quite distinct and is intact. The cytoplasm of the cell contains numerous constituents. Scattered throughout the cell are numerous oval-shaped granules, varying in size from about 0.05 to 0.2 microns. The granules are thus smaller than those of the other mast cells previously described. The granules vary in osmophilia, some being quite dense with an entirely solid matrix, while others are lighter and have clear areas within. Each granule has a distinct limiting membrane. A number of these large granules contained numerous much smaller granules and vesicles. Similar granules and vesicles were scattered throughout the cytoplasm. Round and elongated mitochondria with the usual structure can be seen. Vacuoles found at the cell surface appear to move deeper into the cell and degenerate. Deeply osmophilc amorphous lipid inclusions are present. Abundant smooth-membraned agranular reticulum is present but little endoplasmic reticulum can be seen.

The ascites mastocytoma cells, even though they possess granules are quite different in appearance from normal mouse mast cells,[15] (p.9), rat mast cells,[16] (p.10), human mast cells[19] (p.11), and even the Furth mouse mastocytoma.[17] (p.10). They resemble somewhat the dog mastocytoma cell described by Bloom et al (p.9) in their general configuration, distribution of granules, and active plasma membrane.

Thus, electron microscopy reveals that there is a tremendous species variation in mast cells although this is not as evident in light microscope studies. Indeed, the ascites mast cells look like "typical" mast cells with the light microscope in that they appear full of metachromatic granules other than in the area occupied by the nucleus. The explanation of the difference between the light and electron microscope appearance may be that in the electron microscope we look at a two-dimensional view of very thin horizontal sections which contain granules in only a few areas whereas in the light microscope we see the "composite of a series of thin sections" which collectively contain granules in all areas of the horizontal planes contained in the thin sections so that when viewed from above, as in the light microscope, one gets the impression that the non-nuclear cytoplasm is occupied only by granules. This presupposes that the rest of the material in the cell is essentially transparent.

The Origin of the Mastocytoma Granules

Two of the most interesting and important unanswered questions of amine biology concern the origin of the amine-containing granules and how the amines get from the cell

The nucleus [illegible] cells, even [illegible] large vacuoles are quite at least in [illegible] from normal [illegible] such cells,[15] (p.9), but such cells,[16] (p.10), [illegible] such cells[17] (p.11), and even this [illegible] cytoplasm.[17] (p.14). They readily accepted the [illegible] described by Sibre et al (p.7) in their [illegible] conditions, [illegible] of growth, and active [illegible].

Thus, electron microscopy reveals that there is a [illegible] apparent resolution between cells although this is not as evident in light microscopy studies. Indeed, the cells that cells look like "typical" such cells when the light microscope is that they appear [illegible] reproducible [illegible] the same resolution by the condition. [illegible] of the differences between the light and electron microscopes [illegible] may be due to the [illegible] of [illegible] three-dimensional view of very thin [illegible] sections which contain [illegible] to only a few [illegible] in the light microscope [illegible] to see the "complete" [illegible] of [illegible] "which collectively [illegible] in the [illegible] as the [illegible] views [illegible] in the [illegible] above. [illegible] the light microscope, one [illegible] that the [illegible] cytoplasm is [illegible] only by [illegible]. The [illegible] that the [illegible] of the [illegible] in the cell is [illegible].

The [illegible] of the [illegible]

Two of the most [illegible] and [illegible] [illegible] of [illegible] [illegible] of [illegible] [illegible] the [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] the cell

cytoplasm where they are synthesized from their precursor amino acids by the decarboxylating enzymes[57,58] to the granules where they are stored and yet avoid degredation by the various cytoplasmic or mitochondrial enzymes capable of inactivating these compounds.[59,61] It has been suggested that the amines in the granules are those which have been able to escape degredation,[111] a seemingly wasteful mechanism, or that the amines are bound in some fashion which prevents them from being destroyed while passing from clear cytoplasm to granules.[112]

Pictures of the ascites mastocytoma cells and certain of the atypical granule-containing tissue culture cells provide the basis for a new theory of the origin of the granules of these cells and how they come to contain amines. As previously mentioned, these cells contain an extensive agranular reticulum (which includes the Golgi apparatus) consisting mainly of tubules and sacs seen sometimes in profile as linear arrays parallel to one another but mostly in cross section as microvesicles and microgranules (which are interpreted as microvesicles containing protein, heparin and amines (Fig. 1-3, 13,14). In some areas of the cell, these microvesicles were closely associated with one another but not membrane-bound (Fig.3,G2, G3). These were most commonly seen in the region of the Golgi apparatus (Fig. 3). Elsewhere there are membrane-bound particulates, of the same size and shape as mature amine granules (Figs. 1,2) which contain microvesicles and micro-granules (Fig. 3, G1; Fig. 14, G1). In other particulates of this general nature, the microvesicles and micro-granules

appear to be clumping together (Fig. 3, G1; Fig. 13, G1; Fig. 14, G2, G3). In the final stage of this process, which I believe to be that of granular maturation, the microgranules and microvesicles have coalesced within the membrane-delimited area. The membrane of the final granule is probably a dilated part of the agranular Golgi reticulum which has detached itself from this system.

I further propose that the amines, after being synthesized in the clear cytoplasm gain access to the lumen of the vesicles and cisternae of the Golgi reticulum, part of which is then incorporated into the granules as well as used to form their limiting membrane in the manner just described. This would protect the amines from any degredative enzymes in the mitochondria or cell sap as the amines become granular bound. Green and Day have shown that 30% of the 5-HT and histamine of the solid Dunn-Potter mastocytoma was found in the microsomal fraction.[96] This could represent amines in transit to the granules if the proposed theory is correct.

The granules of normal mouse mast cells have been noted to have a "filamentous structure,"[15] those of normal rat and guinea pig mast cells, a "reticular structure,"[16] and those of normal human mast cells, to consist of "lamellar groups."[19] It has been claimed that the inner part of the granules of the adrenal medulla is in the form of very small granules.[113] The studies of Ball cited by Hagen and Barrnett[114] demonstrate that the granules of the adrenal medulla possess an enzyme characteristic of the microsomes, which are partly made up of agranular reticulum.[115] This led them to speculate that the

protein matrix of the adrenal medullary granules originates in the endoplasmic reticulum (where all proteins are synthesized). Hagen, Barrnett and Lee noted that the large mastocytoma granules of the Furth mastocytoma occurred in vacuole-like spaces in close relationship to the vesicular system of agranular reticulum. They suggest that this "may indicate a probable role of the reticulum in the development of the granules or the laying down of its contents."[17]

Numerous studies with other cell types which produce granules containing secretory material other than amines, suggest that their granules also originiate in, if not from, the agranular reticulum, with which the agranular reticulum is in direct continuity. Wellings, Siegel, Dalton and Felix in a series of papers have extensively studied melanocytes in this regard, demonstrating the progressive maturation of melanin granules within the agranular reticulum.[116-119] Similar mechanisms have been claimed for plasma cells[120] and lactating mouse mammary cells.[121] A morphological and functional association of the reticulum of the pancreatic acinar cell in the development of zymogen granules has been described by Siekevitz and Palade.[122]

This hypothesis that the granules of the Dunn-Potter mastocytoma cells are made from segments of agranular reticulum containing sequestered amines synthesized in the free cytoplasm may be considered an instance of Essner's and Novikoff's thesis that; "Cytomembranes are in a dynamic state of flux, movement and transformation in the living cell and smooth surfaced derivatives of the endoplasmic reticulum become fashioned into the Golgi membranes as the Golgi membranes are

being refashioned into those that delimit secretory vacuoles."[123]

Whether this mechanism is a common one for making amine granules cannot yet be answered. The studies cited concerning the fine structure of the granules of other mast cells [15,16,19] and the adrenal[113] and the chemical nature of the adrenal granules[114] suggests that it may be a widely employed system.

This hypothesis if readily testable by looking for additional enzymatic activities and constituents common to both the granules and the agranular reticulum of the ascites cells, both of which can be isolated in reasonably pure states by procedures which the author developed. These studies are in progress. Electron microscopic autoradiography with radioactive histamine, 5-HT or heparin would be a very elegant way to test the hypothesis.

The Tissue Culture Dunn-Potter X-1-C Mastocytoma Cell

It was thought that distinct advantages would accrue from working with cells from tissue culture rather than with ascites cells aspirated from the mouse peritoneal cavity. The aspirate is really a mixture of neoplastic mast cells, fibroblasts and leuokocytes. It was thought that the cultured cells would provide pure populations of identical mast cells which could be readily obtained in large quantities. Also the tissue culture cells could be easily treated with known concentrations of drugs for known periods of time without the interference provided by the mouse. Similar considerations have made the tissue culture cell the most frequently investigated form of this mastocytoma.

It was thus of great interest to learn that, unlike the ascites cells, the tissue culture cells contained few or no

granules. (Figs. 4-7). While several tissue culture cells definitely possessed granules (Fig. 8), the great majority possessed no cytoplasmic particulates that could be called granules. Those granules that were present in the cultured cells were much smaller than those of the ascitic cells (Fig. 8). This is the only cell that has been described which is functionally a mast cell but which contains no granules. Whether or not it should be called a mast cell is a question of semantics. But the fact that is amines are not in granules is obviously of paramount importance in interpreting experiments on amine biology in these cells.

The tissue culture cells have a relatively undifferentiated structure. They have one or two irregular nuclei with one or more large nucleoli, abundant mitochondria, an extensive agranular reticulum, and pinocytotic vesicles. In these respects, save for more mitochondria and the absence of granules and vacuoles, they are quite like the ascites cells.

The absence of granules in the tissue culture cells sets them apart from the ascites cells and belies their descent from the <u>same cell</u>. The ascites cell line as presently maintained is frequently renewed from the tissue culture cells. Clearly the environment in which these cells divide and grow exerts a great influence on the cells phenotype, on which part of its genetic inheritance is expressed.

The X-1-C cell line is derived from a single cell and is presumably genetically pure. This has led investigators to believe that a population of these tissue culture cells was homogeneous. But, with the elctron microscope, many atypical cells were seen.

[illegible]. (Figs. 6-7). While several fibers without cell-
identifiably connections remained (Fig. 8), the third material
measured no significant contribution that could be called
[illegible]. Those granules that were present in the medium
cell were most evident near the ends of the resting cells
(Fig. 8). This is the only cell that has been described
which is functionally a mast cell but which contains no
granules. Whether or not it should be called a mast cell
is a question of nomenclature, and the fact that it matures
and is granular is evidence of necessary importance
in interpreting experiments on cell biology in these cells.

The three culture cells have a relatively uniform
appearance. They have one of the irregular nuclei with one or
more large nucleoli, abundant chromatin, an extensive
granular cytoplasm, and abundant vacuoles. In these
respects, they are more alike than not. The absence of
granules and vacuoles, that one cells from the resting cells.

The absence of granules in the three culture cells sets
them apart from the resting cells and helps distinguish
from the mast cell. The resting cell line is probably main-
tained in culture because from the tissue culture cells.
Clearly the environment in which these cells develop and grow
exerts a great influence on the cells phenotype, so which
part of the genetic information is expressed.

The T-4-C cell line is derived from a stable cell and
is probably monoclonal. Little has led investigators
to believe that a population of these tissue culture cells
may heterogenous. But, with the electron microscope, has
displayed cells more than.

As was mentioned, a few cells were seen which were identical in all respects, save for the appearance of membrane bound inclusions of smaller size than mitochondria, but lacking an internal structure, which could be amine granules or degenerated mitochondria (Fig. 8). Just why these cells and no others produce granules cannot yet be answered.

A few cells were seen which contained bizarre "multi-vesiculated dense bodies" which look very much like the lysosomes described by de Duve (Fig. 9).[124] These were seen very infrequently in the ordinary tissue culture mastocytoma cells.

The most interesting atypical cell was seen in a reserpine-treated group of cells. The basic morphology of this cell could not be ascribed to the effects of reserpine since it was the only one of tens of thousands of reserpine-treated cells that had this appearance. This cell bore a striking resemblance to the normal mouse mast cells described by Rogers (p. 9, Figs. 10-12). Both cells possess about the same number of granules, each surrounded by a clear area and located at the periphery of the cell. The cytoplasm of both cells is relatively undifferentiated. In the reserpine-treated mastocytoma cell, some of the granules are being released through the cell membrane. This will be discussed shortly. The similarities between this "atypical" neoplastic mouse mast cell and the normal mouse mast cell prompt the speculation that the genetic material of the neoplastic cell line still possesses the information to produce a cell near-normal in appearance on occasion.

At the conclusion, a few cells were seen which were totally lysed in the presence, even in the complement of receptors bound immunoglobulins of similar size than antimonocytes, but lacking on receptor specificity, which could be seen (precision of approximately 1000-fold) (Fig. 7). Such cells these cells and to observe previous studies cannot yet be answered.

A few cells were seen which contained intracellular "multivesicular bodies" which look very much like the lysosome described by de Duve (Fig. 7). These were seen very infrequently in the cytotoxic direct culture preparations cells.

The most interesting observation until now seen in a receptors-treated cross of cells. The basic morphology of this cell could not be ascribed to the effects of receptor alone. It was the only one of kind of treatment of receptors-treated cells from but this preparation. This cell bore a striking resemblance to the normal horse heart cells described by Jensen (, Figs. 1c-1d). Most cells possess about the same number of granules, each surrounded by a lipid core and located at the periphery of the cell. The cytoplasm of such cells is relatively undifferentiated. In the receptors-treated monocytes cell, some of the granules are being released through the cell membrane. This will be discussed shortly. The distinctive feature with "apoptosis" morphologic cross such cell and the normal horse heart cell proper the cytoplasmic that the granule membrane of the monocytic cell like that which potentiates the interaction to produce a cell mere-normal in appearance to neighbors.

Another very interesting variant which was seen only once was a cell three or four times as large as large as any other cell, with four nuclei, and a cytoplasm densely packed with granules of varying sizes and density, vesicles and mitochondria. (Figs. 13, 14). Significantly, this tissue culture cell which possessed granules demonstrated what I interpret as early forms of granules, made up of agranular reticulum, and identical to those seen more frequently in the ascites cells.

A number of cells were seen whose cytoplasm was occupied mainly by large clear area which could have been granules with a sparse matrix. (Fig. 15). These cells resembled the normal rat and hamster mast cells described by Smith and Lewis.[16]

It can be stated with conviction that the failure to see granules in most of the cultured cells in electron micrographs is not an artifact of preparation. The same technique of osmium tetroxide fixation, alcohol dehydration and embedding in methacrylate or epon have been successfully used by all investigators who have published electron micographs of mast cells.[14-17,19] The same technique demonstrated granules in the ascites Dunn-Potter mastocytoma cells. In addition, the fine structure of the tissue culture cells was excellently preserved.

It is possible, but very unlikely, that all the amines are present in the granules of the few cells with granules and that the great majority of cells possess neither granules

Bacteria and fibroblasts cultured within are seen only once as a cell cluster or thin layer or layers as large as any other cell, with their nuclei, in a stringlike manner made up of clusters of various sizes and shapes, resulting and microscopically, (Fig. 12, 13). Not different, while tissue culture and other research problems involved work I happened to carry, terms of growth, medium or culture medium, and tissues to those were just frequently in the another cells.

A number of cells were seen under microscope was occupied mainly by large clear and vital cells have been growing with a mature nucleus, (Fig. 14). There is no modified the normal cell and because some cells degenerated by nuclei and lysis.[10]

It can be stated also generally that the failure to use growing in most of the cultured cells in direction microscopic is not on cellular of production. The most technique of cells tetanoide fibrosis, alcohol coagulation and embedding in paraffin or cross-section been successfully used by all investigators who have published direct stain-pages of tissue cells.[12,13] The most backgrounds demonstrated growing in the nucleus than cytoplasm cells. In addition, the fine structure of the tissue or fibre cells are microscopically analyzed.

It is possible, but very unlikely, that all the tissue are present in the growth of the fibre cells with cytoplasm and that the usual majority of cells possess neither growth

or amines. This would necessitate an enormously high concentration of amines in these granules, approximately 100,000 times the concentration of amines in the ascites tumor cells based on the amine concentration of each cell type, the percentage of cells with granules and the number of granules per cell.

It is clear then that most of the amines in these cells are not granular bound unless they are present in mitochondria. Certain evidence makes this unusual possibility at least worth considering, even though every amine-containing cell studied to date has amine granules which are distinct from mitochondria.[111] First, these cells are unusually rich in mitochondria; whereas the ascites cells possess few mitochondria and many granules, the reverse is true of the tissue culture cells. Second, density gradient centrifugation of the tissue culture cell large granule fraction, which sedimented between 700g and 10,000g in thirty minutes and contained 53% of the 5-HT of the cultured mast cells gave results compatible with the amines being in the mitochondria: 49% of this fraction's 5-HT was in a sub-fraction which contained 60% of its succinoxidase activity (located in the mitochondria)[118] while another 43% of the 5-HT was found in a sub-fraction which contained 24.5% of the succinoxidase activity.[6] Thus, Most of the amines and succinoxidase were in the same sub-fractions implying that they might well be located in the same sub-cellular particle. My attempts to check this by electron microscopy will be reported and discussed in full, later, but it should be mentioned here that they are consistent with the proposition that the amines are located in

or embryo. This would necessitate an unusually high concentration of nutrients in these samples, approximately 100,000 times the concentration of nutrients in the media. Based on the volume of each cell, the percentage of cells with pressure and the number of pure has per cell.

It is clear from this most of the cells in these cells are not primarily owned unless they are present in photosynthesis. Certain evidence makes this unusual possibility at least doubtful, even though every cells-containing cell should to data has some pressure which are isolated from attachment. Fig. 1 Fig. These cells are usually seen in bibliography; whereas the another cells possess low microscopic and only pressure, the reverse is true of the blue algae cells. Second, density pressure contribution of the blue algae cells large pressure increase, which ranged between 700 and 10,000 in thirty minutes and contained 32% of the 7-10 of the cultures that cells were usually compatible with the cells being in the microscopic: 10% of this increase 5-HT was in a sub-fraction which contained 90% of the pressure activity (pressed in the microscopic) [18] while another 10% of the 7-10 was found in a sub-fraction which contained 90.2% of the pressure activity, as the same sub-fractions i trying that they might well be located in the same sub-cellular particle. It attempts to check this by electron microscopy will be repeated and discussed in full later, but it should be mentioned here that they are consistent with the proposition that the cells are located in

the mitochondria. The data on the cultured cells just presented should be contrasted with the author's studies using density gradient centrifugation of the large granule fraction of the ascites cells which were done several years before the work just presented. With the ascites cells, the author was able to obtain one sub-fraction of the large granule fraction which contained 56% of the total succinoxidase activity and no 5-HT at all, while another sub-fraction had 100% of the 5-HT and histamine and only 28% of the succinoxidase activity. This is at least suggestive that in the ascites cells there are amine storage granules which are not mitochondria and indeed it is the ascites cells which do contain many granules when viewed in the electron microscope. Some mitochondria have been found to contain phosphatides and cerebroside sulfate, both of which have been implicated in amine binding.[111] The chief objection to 5-HT being stored in mitochondria would be that the enzyme which oxidizes 5-HT, monoamine oxidase (MAO) is a mitochondrial enzyme.[125] The author's prolonged studies with sensitive MAO assay techniques failed to find any MAO activity in the Dunn-Potter mastocytoma. The same is true for histaminase, the enzyme which oxidizes histamine. Thus, both these amines could be stored in mitochondria, whereas this would be impossible for the usual cell which contains biogenic amines and the enzymes which degrade them.

It is very hard to conceive of the mitochondria being used to store amines. As was mentioned, all amine-containing cells studied to date have amine granules distinct from

the chloroplasts. The data on the cultured cells just mentioned should be considered with the evidence publish using density gradient centrifugation of the large pressure fraction of the amoeba cells which were done several years before the work just presented. With the amoeba cells, the author was able to obtain one sub-fraction of the large pressure fraction which contained 55% of the total succinoxidase activity and no D-LD at all, while another sub-fraction had 100% of the D-LD and succinoxidase and only 45% of the succinoxidase activity. This is at least suggestive that in the amoeba cells there are mitochondria containing which are not mitochondria and indeed is in the amoeba cells which do contain many granules when viewed in the electron microscope. Some chlorophyll have been found to contain phosphatidylcholine and cerebroside sulfate, both of which have been implicated in energy binding.[11] The added evidence to D-LD being found in chlorophyll would be that the enzyme which oxidizes D-LD, monoamine oxidase (MAO) is a mitochondrial enzyme.[12] The author's unchanged studies with amoeba PAC heavy fraction failed to find any MAO activity in the succinoxidase preparations. The case is true for chlorophyll, the enzyme which oxidizes D-LD. Thus, both these enzymes could be found in chlorophyll, whereas this would be impossible from the usual cell which contains chlorophyll and the enzyme which degrades them.

It is very hard to conceive of the mitochondria being used to store energy. As was mentioned, all amoeba-containing cells studied to date have some granules distinct from

mitochondria. The role of the mitochondria in energy metabolism hardly makes it suitable to act as a storage particle. The mechanism of amine storage I just postulated for the Dunn-Potter ascites cells would be ruled out. Why the tissue culture cells should store their amines in mitochondria is not clear. Much more work will have to be done to settle this problem. At this point, all we can say is that the experimental evidence available concerning the tissue culture cells is consistent with the possibility that in these cells the mitochondria contain histamine and 5-HT but that past experience with other amine-containing cells makes this a highly unlikely possibility.

It is possible that the amines or any complexes they may be bound in, are free in the cytoplasm. Furano's finding of the particulate nature of the amines of these cells[6] may be spurious and represent cytoplasmic amines adsorbed on particles after homogenization. The absence of monoamine oxidase and histaminase in these cells which I demonstrated would permit the amines to be non-sequestered.

Effects of Reserpine on the Tissue Culture Mastocytoma Cells

It was a difficult decision to decide between using the ascites cells which possess granules and the tissue culture cells which possess essentially none to investigate the process of amine release with reserpine. I decided to use the tissue culture cells because this is the cell that several other investigators are using to investigate the release process

microscopic. The role of the chromosomes in energy transmission [illegible] is probably to act as a storage [illegible]. The mechanism of cellular division I have considered for the [illegible] cells would be rather [illegible]. May the tissue culture cells should show their failure to [illegible] in such cases. Much more work will have to be done to settle this problem. In this point, all we can say is that the experimental evidence [illegible] necessary the [illegible] cultures cells is consistent with the [illegible] that in [illegible] cells the [illegible] contain [illegible] and [illegible] but [illegible] [illegible] with other [illegible] cells seems [illegible] a [illegible] [illegible].

It is [illegible] that the [illegible] or any [illegible] they may be [illegible] in the [illegible]. [illegible] the [illegible] of the [illegible] of the [illegible] of [illegible] may be [illegible] and [illegible] [illegible] or [illegible] [illegible]. The [illegible] of [illegible] [illegible] and [illegible] in these cells which [illegible] [illegible] [illegible] the [illegible] to be [illegible].

[illegible] in the Tissue Culture [illegible]

It was a [illegible] decision to decide between using the [illegible] cells which [illegible] and the tissue culture [illegible] cells which possess [illegible] [illegible] the [illegible] of cells [illegible] with [illegible]. I decided to use the [illegible] culture cells because [illegible] in the cell [illegible] [illegible] [illegible] are [illegible] to [illegible] the [illegible]

with biochemical and pharmacologic techniques. The opportunity to correlate their data with morphologic changes in the cell was decisive.

After being treate d with reserpine at concentrations of 10^{-7} for twenty-four hours, the tissue culture mastocytoma cells were found to contain less than 0.1 gamma of 5-HT per 10^{8} cells, less than 1% of the control levels. Similarly, the cells had lost the greater part of their histamine content. The cells treated with 10^{-9}M reserpine had approximately 50% of their original levels of 5-HT and histamine at the end of this time.[69] When these cells were looked at with the electron microscope after suitable preparation, a number of different reactions to the drug treatment were noted. They may represent different stages of the release process so that all cells at one time may have gone through the stages seen. The cells were observed only at the end of twenty-four hours when there was no further measurable release at both concentrations of the drug studied. It is obviously of interest to study the cells at earlier times when the process is not yet complete. Thus, the cells to be described are cells who have already released their amines, some perhaps with the cellular changes that brought about still present, others with the cell not showing any unusual features, but lacking amines.

The outstanding change in cell morphology noted was the development of varying numbers of "multivesiculated dense bodies" in a large percentage of the cells. (Figs. 16,17). Some cells possessed as many as thirty of these bodies (Fig.17).

They are 0.2 microns or oval in shape, and characteristically densely osmophilic with internal discrete clear areas. The appearance of these bodies suggests that they are lysosomes, the enzyme-containing particulates de Duve discovered in liver and subsequently found in a number of the tissues.[124] The multivesiculated dense bodies were almost never found in the untreated cells. More were found in the cells treated with 10^{-9} M than in those treated with 10^{-7} M reserpine, perhaps because the release process was incomplete at the lower concentration.

The chemical nature of these particles together with what is known about amine-binding and release suggests that the appearance of these particles in reserpine-treated, amine-releasing cells is not simply coincidental; rather evidence can be marshalled for the theory that reserpine brings about amine release in these mastocytoma cells by "inducing" the formation of lysosomes, whose enzymatic components could serve as the actual means of bringing about amine release.

First, let us consider the nature of the lysosomes. Their discovery is credited to de Duve who found he could separate by centrifugation a subcellular fraction from liver, rich in acid phosphatase and poor in mitochondrial enzymes.[124] These particles have since been found to contain at least ten hydrolases which have an acid pH optimum: acid phosphatase, the cathepsins A,B, and C, phosphoprotein phosphatase, mannosidase, acid ribonuclease, acid deoxyribonuclease, B-galactosidase, B-glucuronidase and arylsulfatase A and B.[124]

As will be indicated, several of these enzymes are strong candidates for a role in the release of amines.

The lysosomes of the liver are fairly polymorphic. Some "appear to be solid and are surrounded by a single membrane; others show one or more internal cavities, sometimes lined with a broad layer of denser material, or contain clumps of such material." Most of them have a fine granular structure. Lysosomes of other tissues have somewhat different size and shape. They have been found in practically all tissues.[126] If we are correct in considering the "multivesiculated dense bodies" as lysosomes, this will be the first identification of these structures in mast cells. Enzymatic studies are in progress to definitely identify these bodies as lysosomes. These cannot be reported as yet. However, additional morphologic evidence will be presented subsequently which tends to support their identification as lysosomes.

To understand the proposal that lysosomal enzymes are important in amine release, it is first necessary to review some of what is known about amine binding. The most information available on this subject pertains to the catecholamines stored in the granules of the adrenal medulla. All evidence points to an analagous mechanism existing in all amine-containing cells, including the mast cells.[111]

Hillarp et al. has shown that in the adrenal medulla the sympathomimetic amines are found in granules[127] which others have shown are bounded by a membrane.[128,113] Hillarp showed that amines, adenosine phosphates, proteins and lipids represent practically the entire content of solids in the

to will be indicated, because of local anatomy and alternate candidates for a molecule has original of action.

The presence of the liver has been [illegible]. Some "organs" to be critical are surrounded by a single membrane; others show one or more internal cavities, sometimes lined with a broad layer of vacuolated cytoplasm, or contain clumps of such material." Much of these have a fine granular appearance, features of other tissues have suggested different size and shape. They have been found in evolutionally all stages.[10] It has been proposed to establishing the "multinucleated cell bodies" as lipochrome, but not in the final identification of these structures by just cells. Regressive station are in process to determine identify where follow as lipochrome. These cannot be completed as yet. However, additional cytologic evidence will be presented subsequently which tends to support their identification as lipochrome.

In conclusion has proved that lipochrome cytoplasm are important in solar injury. It is fair assuming in review some of that is known about cells of [illegible]. The most [illegible] available on this subject pertains to the [illegible] in the creation of the adrenal medulla. All evidence points to an [illegible] origin relative to all chromaffin-containing cells, including the [illegible] cells.[11]

Milian et al. has given stat on the adrenal medulla the epithelioid cells are found in [illegible] which [illegible] have been [illegible] by a [illegible].[12,13] Ultimately [illegible] thin walled, [illegible], [illegible] and lipoid [illegible] [illegible] in the [illegible]

granules.[129] He and his colleagues also showed that the granules of the adrenal medullary cells store catechol amines and ATP in nearly equivalent amounts[130,131] and that stimulation of the medulla *in vivo* causes a drop in the ATP that is proportional to the decrease in catechol amines.[132,133] Sheep treated with reserpine released amines and adenosine phosphates from their adrenal medulla at the same rate.[134] Hillarp believed he had demonstrated ATPase activity in the adrenal medullary granules.[135] This finding was disputed by others who claimed Hillarp's granule fraction was contaminated with mitochondria and that pure fractions of granules contain no ATPase.[136] Nevertheless, the presence of ATPase in the granules is still an open question because of the difficulty of demonstrating this enzyme. Hillarp suggested that amine release is brought about by the dephosphorylation of ATP to which the amines are probably bound, by the ATPase of the granules which is activated, either directly or indirectly, by the agent which triggers release.[135]

The lysosomal enzymes might work independent of, or together with granular ATPase or other intragranular mechanisms of release. Let us consider an independent mode of action first. The action of the lysosomal enzymes might be to degrade the anion which the amines are bound to - in the mast cell this is probably either heparin or ATP.[1,111] The lysosomes possess both sulfatases and phosphatases[124] although it has not been proven that heparin and ATP are

substrates of these enzymes. In the tissue culture mastocytoma cells where the amines may be in the cytoplasm, the action of these enzymes on the amine complexes could occur directly; in cells where amines are in granules, the enzymes would first have to enter the granules. The lysosomal enzyme (s) might increase the permeability of, or otherwise alter strategic membranes with the cell, perhaps either the granule membrane or the cell membrane. These membranes are basically lipoprotein in nature and the lysosomes possess the necessary equipment (phosphatases, proteinases) to bring about their disruption. After disruption, amine release may occur simply by diffusion because a previously impermeable barrier has now been removed, or contact of lysosomal degredative enzymes and the anions bound to the amines may be promoted. Another possibility is that disruption of the granule membrane may change conditions with the granule, such as the pH or the concentration of various ions within the granule and thus modify the activity of the enzymes present in these granules. As has already been mentioned, there is evidence for an ATpase in the granules.[126,135] One of the important questions regarding the significance of granule ATPase in the release of amines was the mechanism by which it might be activated at the time of release. The change in local conditions that might occur as the result of the action of lysosomal enzymes may be the answer.

It might be argued that the appearance of lysosomes in these cells is an indication of cell death because the lysosomes

[illegible]

have been implicated in this process.[119] Against this is the finding that the reserpine-treated cells will resynthesize their amines, divide and grow when placed in fresh media.

It is clear that no such lysosomal-mediated explanation of the mode of action of reserpine is possible when one is dealing with isolated amine particles *in vitro*. But it is not at all necessary that these two phenomena have a common mechanism. It has already been pointed out that *in vitro* reserpine possesses certain properties such as an inhibition of amine release which are never seen *in vivo*.

It is quite possible, of course, that the increase in lysosomes following reserpine treatment, has nothing to do with amine release. Other morphologic differences between some few reserpine-treated cells and untreated cells were seen. These will now be described.

At the very outset of these experiments on the neoplastic mouse mast cells, I hoped to see the process of granule release in reserpine-treated cells. The almost invariable absence of granules in these highly dedifferentiated cells generally frustrated this desire. How surprising it was, then, to find one cell, which had been treated with reserpine, in which a number of granules were passing through or had already traversed the cell membrane, and whose general morphology bore a striking resemblance to that of a normal mouse mast cell as described by Rogers (Figs. 10-12 p.9).[14] A possible interpretation would be that this cell had regained some of its normal characteristics and was responding as a normal granule-filled mast cell would, that is, with

granule release. Some reserpine-treated typical tissue culture cells were seen which had cytoplasmic inclusions passing through the cell membrane (Fig. 18). This was occasionally seen in untreated cells. I am presently investigating the effects of reserpine on the ascite mastocytoma cells which do possess granules.

Reserpine treatment produced a group of cells which suggested another mode of amine release. These cells had large membrane-bound areas with a very sparse matrix. (Fig. 19). In several of these cells, these areas were located at the cell surface with a partially disrupted segment of the plasma membrane as one of its borders. Other such areas were often seen deeper within these cells. It is possible that discharge of cellular contents had taken place at the cell surface, leaving behind amorphous membrane-bound inclusions that then detached themselves from the cell surface to migrate back into the middle of the cell. What makes this of particular interest is that De Robertis and Vaz Ferreira described a similar occurrence in the adrenal medulla of rabbits after stimulation of the spalnchnic nerve.[137] Their observations suggested that the secretion of the catechol amines involved an increase in size but a decrease in electron density of the granules and their attachment to the cell surface. The granules then released their contents at the cell surface and were left as folded membranes attached to the cell surface. Furthermore, Wellings and De Ome observed that in lactating mouse mammary cells vacuoles

containing milk protein granules appeared to open at the cell surface and release their contents into the alveolar lumen. Similar methods of secretion have been observed in many other tissues. An analogous process might be happening in the tissue culture mastocytoma cell, differing in that a whole region of the cell enclosed within a membrane, probably part of the agranular reticulum is being discharged. If the amines are located in the cytoplasm of these cells this would effectively accomplish amine release. This cell type was not seen in any of the untreated cells. It is unlikely that it represents vacuole formation at the cell surface, although a secondary result of such a process might be imbibition of extracellular fluid.

At least a half of the cells seen after treatment with both 10^{-7}M and 10^{-9}M showed no morphologic difference from untreated cells, despite the fact that the cells treated with 10^{-7}M possessed less than 1% of their normal amine concentration. This indicates that release can occur without any visible change in the cells morphology, or that the changes which brought about or accompanied release in these cells was no longer evident. It is impossible to make a choice between these two interpretations, although the latter possibility seems more likely.

It would be nice to fit all these findings into a single interpretation that would encompass them all. Since the untreated cells are clearly polymorphic, we are not really required to do so. Reserpine may act differently on

each different cell type present. This clearly seems to be the case in the cells with granules where reserpine produced release of the granules. It is thus possible that these findings, the induction of what appear to be lysosomes, the release of granules, and the release of cell cytoplasm from membrane-bound areas are phenomena seen only in these atypical neoplastic cells. Nevertheless, they do suggest new experiments which should and can be done on other tissues such as the adrenal medulla and the central nervous system.

Some suggestions may be offered about the origin of and additional activities of the "multivesiculated dense bodies" which both tend to support that they are lysosomes and indicate how reserpine promotes their formation. At the same time they contribute in a small way to what is known about the origin of and the activity of the lysosomes. In Figs. 16 and 17, in the midst of areas packed with the multivesiculated dense bodies, we see membrane-bound vacoules filled with additional segments of membrane and clumps of osmophilc matter. They appear to be early stages of the osmophilic dense bodies. In Fig. 17, we see the dense bodies seeming to ingest mitochondria. This phenomenon has been described previously by Novikoff[138] and the resulting inclusion has been called a "cytolysome".[19] These micrographs suggest that the lysosomes are formed in dilitations of the agranular reticulum, as suggested by Novikoff[140] by ingesting cellular material and that once formed

the lysosomes can incorporate other cell contents such as mitochondria.

Effects of Chlorpromazine on the Dunn-Potter Tissue Culture Mastocytoma Cells

Until recently most of the experimental studies of the biochemical basis for chlorpromazine's central nervous system depressant or tranquilizing action were concerned with its activity as an inhibitor of respiratory enzymes or oxidative phosphorylation.[141] *In vitro* effects could be demonstrated on these systems only by using concentrations of the drug hundreds of times greater than those achieved *in vivo*.[141] Recent experiments which were reviewed in the Introduction (p. 91-92) have led to the hypothesis that the primary effect of chlorpromazine is on various cell membrane phenomena,[82] particularly those of the mitochondria.[83-93]

Studies with the mast cells treated with chlorpromazine at 10^{-5} M for twelve hours demonstrate a morphologic derangement of mitochondrial structure which seems to result from the action of this drug. (Figs. 20-22). In the untreated mastocytoma cells the outer limiting membrane of the mitochondria is smooth contoured and continuous, whereas the inner one folds inward periodically to form the incomplete septa that form the cristae. The two leaves of the crista usually run straight and parallel and are separated by a narrow interspace of uniform width (Figs. 4-7, 23).[142] The mitochondria of the chlorpromazine treated cells frequently have highly disrupted outer membranes. Cristae are

seldom perpendicular to the limiting mitochondrial membrane but are instead parallel or come off at irregular angles. Some mitochondria appear compartmentalized by crista running in a number of directions. Other mitochondria consist of whorls of membranes, like the myelin of a nerve sheath. There are numerous elongated unusually shaped mitochondria in the treated cells. There are very few normal appearing mitochondria in most of the treated cells (Figs. 20-22). This is to be contrasted with the appearance of "multi-vesiculated dense bodies" in about a quarter of the cells after reserpine treatment.

The morphological effects of chlorpromazine on mitochondrial membranes could be due to the same chemical changes which produce inhibition of mitochondrial swelling in hypotonic solutions, etc. (p. 91-92),[85-93] or the reverse could be true and the chemical effects might be due to the morphological effects which have some other origin. These morphological effects could explain the inhibition of mitochondrial respiratory enzymes which are known to be dependent upon an intact mitochondrial structure and to involve a series of enzymes located in precise order along the cristae.[143] The bizarre mitochondrial forms produced by chlorpromazine should interfere with these delicate functions to a great extent. On the other hand, the enzymatic inhibition could be the cause of the morphologic aberrations. Regardless, this work has established an

effect of chlorpromazine on mitochondrial membranes that is well suited for experimental analysis.

Giarman has established that this dose of chlorpromazine release significant amounts of 5-HT from the tissue culture cells.[144] As was discussed previously, it is possible that the tissue culture cells store their amines in mitochondria. If so, the release of amines by chlorpromazine may be related to the mitochondrial structural changes just discussed as cause or effect. The increased disruption of the plasma membrane of the mastocytoma cells may also be involved. There was no evidence of lysosome formation, granule release or release of cytoplasm in the chlorpromazine-treated cells, as was seen in the reserpine-treated cells.

The Large Granule Fraction of the Tissue Culture Cells

A large granule fraction was isolated from the tissue culture cells after homogenization in 0.3M sucrose. This fraction was layered on a density gradient according to the method of Furano[6] but no separation was achieved. The only tissue evident after centrifugation was on the bottom of the centrifuge tube. This material was prepared for electron microscopy which revealed that it consisted largely of distorted mitochondria (Fig. 24). Very few granules were noted. It is clear that these cells possess very few if any granules.

effect of an increase in intracellular concentration is well suited for controlling a function.

Cannon has established that a rise of acetylcholine causes glycogenolysis [illegible] from the liver without cells.[illegible] In our laboratory, it is known that the liver cells have been shown to be glycogenolytic. If so, the answer is seldom or other concerns may be attributed to the intracellular acetylcholine [illegible] to be in a cause or action. The increased [illegible] of the glycogenolytic effect of the acetylcholine cells may also be involved. There has no evidence of [illegible] [illegible] or release or adrenalin in the adrenergic [illegible] cells, as was done in the [illegible] treated cells.

The Liver Cells in Response to the Cholinergic Cells

A [illegible] [illegible] was [illegible] from the [illegible] cellular cells [illegible] in a "[illegible] [illegible]". This [illegible] was [illegible] of a [illegible] [illegible] according to the [illegible] of "[illegible]" [illegible] [illegible] activated. The [illegible] [illegible] [illegible] [illegible] in the [illegible] of the [illegible] [illegible]. This [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible]. Very few [illegible] were [illegible]. It is [illegible] that [illegible] [illegible] [illegible] [illegible] for [illegible] [illegible].

Isolation of Mast Cell Granules from the Ascites Cells

The purpose of this work was to isolate the amine and heparin containing granules of these cells so that accurate chemical studies of their nature could be carried out. These studies were carried out before electron microscopy revealed that the ascites cells, but not the tissue culture cells possessed such granules, so the choice was a fortuitous one.

Initially, the cells were vigorously homogenized with the Potter-Eveljhem apparatus, followed by removal of unbroken cells, nuclei and debris by centrifugation. A large granule fraction is then sedimented from this cell and nucleus free homogenate by centrifugation and the distribution of amines, heparin and succinoxidase between the large granule fraction and supernatant determined. The results of one experiment are given in Table 1. They are representative of many similar experiments.

Table 1

The Distribution of Histamine, 5-HT and Heparin in Fractions Derived from Mastocytoma Cells by Differential Centrifugation

	Nucleus-free Homogenate	Large Granule Fraction	Supernatant
Histamine	63	53	15
5-HT	180	66	51
Heparin	100,000	67,500	31,500
Succinoxidase	7,600	5,400	1,750

Histamine and 5-HT are expressed as micrograms. Heparin is expressed as total counts. Succinoxidase is expressed as change in optical density under the assay conditions described in the section on Methods.

This demonstrates that the greater part of the histamine, heparin and succinoxidase is particulate in nature. Only about 35% of the 5-HT of the nucleus free homogenate was found in the large granule fraction. Some 28% was in the supernatant. 37% of the 5-HT could not be accounted for and is presumed to be non-enzymatically destroyed since, as will be discussed shortly, no enzyme system capable of degrading 5-HT has ever been found in these cells. Values for the amount of 5-HT which could be recovered in the particulate fraction varied between 30 and 45% in numerous experiments. But the 37% of the 5-HT of the nucleus free homogenate which could not be accounted for, as well as some or all of the supernatant 5-HT might originally have been in the granules. Undoubtedly, much 5-HT is released from the granules during the arduous process of homogenization needed to disrupt these cells. The 5-HT must be more easily

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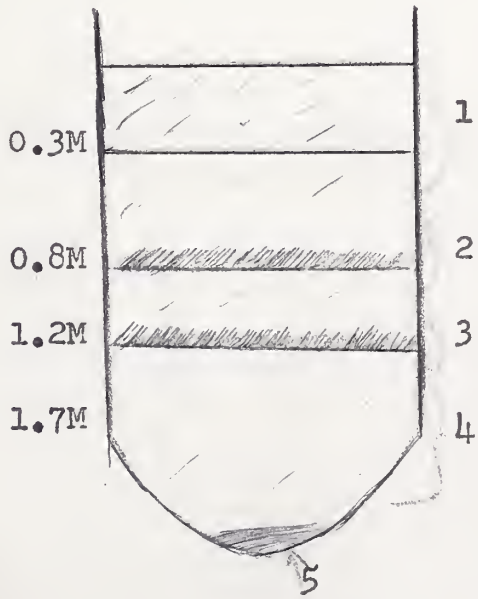
removable by mechanical methods from the granules than heparin or histamine. Hagen, Barmett and Lee have shown the granules of the Furth mastocytoma easily lose their amines in isolation procedures.[17] Of the 5-HT of the nucleus-free homogenate which could be accounted for either in the particulate form or in the supernatant, 60% was particulate (Table 1).

The large granule fraction is then subject to density gradient centrifugation as previously described. After centrifugation, two distinct bands of tissues are evident; (Text Fig. 1) the first is at the border of the 0.8M and 1.2M sucrose solutions and the second is at the border of the 1.2M and 1.8M sucrose solutions. There is a small precipitate at the bottom of the tube.

Text Fig. 1



Five fractions are taken with the aid of the tube cutting device already described and aliquots analyzed for 5-HT, histamine, heparin and succinoxidase. The results are described in Table 2.

Table 2

Distribution of 5-HT, Histamine, Heparin and Succinoxidase in the Five Fractions Obtained From Density Gradient Centrifugation of the Large Granule Fraction.

Fraction	5-HT		Histamine		Heparin		Succinoxidase	
	Units	%	Units	%	Units	%	Units	%
1	0	0	0	0	15,000	26	700	16
2	0	0	0	0	0	0	2400	56
3	63	100	12	100	30,000	53	1100	28
4	0	0	0	0	7,600	13	0	0
5	0	0	0	0	4,500	8	0	0

5-HT and histamine are expressed as micrograms. Succinoxidase is expressed as change in optical density under the assay conditions already described. Heparin is expressed as total counts.

All of the 5-HT and histamine and 53% of the heparin is in fraction three, which is the material which did not sediment below 1.7M sucrose. This fraction contained 28% of the succinoxidase activity as well. Fraction 2 contained 56% of the succinoxidase activity and no 5-HT, histamine, or heparin.

Fraction 2 was resuspended over a second density gradient consisting of 2.5cc 0.8M sucrose, 5.0cc 1.5M sucrose, 7.5cc 1.6M sucrose and 5.0cc of 1.7M sucrose and centrifuged for an hour at 25,000g. A single band was evident at the level of the 1.5M sucrose layer. This was found to contain nearly all of the histamine, heparin,

TABLE 1

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Treatment	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
1	0	0	0	0	10,000	20
2	0	0	0	0	0	0
3	25	100	25	100	10,000	25
4	0	0	0	0	7,000	25
5	0	0	0	0	8,000	0

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5-HT and succinoxidase that had been layered over the gradient indicating no further separation was achieved by the second gradient.

This should not be constructed as an indication that in the ascites cells, the amine-containing particles are identical with some of the mitochondria. Had this second gradient produced a fraction with succinoxidase activity, but containing no amines, we could have definitely ruled out the identity of these particles. The only fair interpretation of the data is that there is in these cells a population of mitochondria which behaves identically to amine-containing particulates during certain centrifugation procedures. Electron microscopy of the intact cells suggests that the amine granules and mitochondria are different. Electron microscopy of the isolated fractions will be done shortly and should help settle the problem. At the present stage of purification, the amine-containing particles in fraction 3 are not sufficiently uncontaminated by mitochondria to permit the precise studies needed to characterize the granules chemically. Other techniques will be employed to improve the separation.

Monoamine Oxidase and Histaminase (Diamine Oxidase)

The attempts to detect the presence of these enzymes by liberation of ammonia from substrates such as tyramine, 5-HT and histamine by the method of Cotzias and Greenough[107] met with no success. In a few experiments on monoamine oxidase, small amounts of ammonia were produced, but the production

was not inhibited by known monoamine oxidase inhibitors such as iproniazid. No evidence for histaminase was found.

The sensitive spectrophotometric assay for monoamine oxidase[110] failed to reveal any enzyme present in mitochondria from large numbers of the ascites, as well as the solid tumor cells. It could be calculated from the number of cells used that if they do possess this enzyme, they contain less than 1/300th the amount present in mouse liver.

The absence of both MAO and histaminase in these cells is to be contrasted with the presence of MAO and absence of histaminase in the Furth mastocytoma.[17] It would be very interesting to determine if normal mast cells possess these enzymes. Green points out that cells which contain biogenic amines, almost invariably contain enzymes which inactivate them.[111] Thus, these amines must normally be protected from the degredative enzymes, usually by granule sequestration. The absence of these enzymes in the Dunn-Potter mastocytoma cells allowes the cell wide latitude in its handling of the amines, and it thus is less surprising that the tissue culture form of this tumor possesses no granules, but may store its amines either in the mitochondria or the free cytoplasm as previously discussed. No catabolism of histamine or 5-HT by any enzyme system has ever been found in this tumor, though it has been extensively looked for.[30,43] This opens up the interesting question of what prevents the cells from continually accumulating amines.

SUMMARY

1. The basic structure of the Dunn-Potter mastocytoma X-1-C cells grown in tissue culture and the mouse peritoneum (Ascitic cells) as seen in the electron microscope, is described. These cells are 7-15 millimicrons in size, possess in common 1-2 nuclei usually irregular in shape, a plasma membrane, a well-developed agranular reticulum, but sparse endoplasmic reticulum and lipid inclusions. The ascites cells possess large vacuoles which do not appear in the tissue culture cells.

2. The ascites cells possess varying numbers of oval granules about 0.05 and 0.2 microns. They have few mitochondria.

3. The tissue culture cells possess few or no granules and large numbers of mitochondria. The possible storage of amines in these cells in mitochondria is discussed. The polymorphism of the tissue culture cells is described.

4. A new theory for the origin of the amine granules and how they get the amines they contain, is proposed and supported. Collections of microvesicles and micogranules of the agranular reticulum appear to be reorganizing within dilitations of the agranular reticulum into mature granules. Chemical assays and evidence from the literature are advanced in supports of this interpretation.

5. It is proposed that the amines after synthesis by the cytoplasmic decarboxylases are secreted into the granular reticulum which then is formed into the granules.

6. Treatment of the tissue culture cells with reserpine at 10^{-7}M and 10^{-9}M for 24 hours, which releases approximately 100% and 50% of the histamine and 5-HT of these cells respectively results in the formation of "osmophilic dense bodies" in many of the cells. It is proposed that these osmophilic dense bodies are lysosomes. A number of possible mechanism whereby the lysosomes might bring about amine release are discussed.

7. The release of granules in an unusual reserpine-treated cell which strikingly resembled normal mouse mast cells is described.

8. The possible relationship to amine release of certain membrane-enclosed clear areas of cytoplasm in the reserpine-treated cells is discussed.

9. Gross derangement of the structure of the mitochondria of chlorpromazine-treated tissue culture cells is illustrated.

10. The morphology of the large granule fraction of the tissue culture cells is described. It consists mainly of mitochondria.

11. The separation of the mitochondria and amine granules of the tissue culture cells by density gradient contrifugation is described. One fraction contained 56% of the mitochondrial enzyme activity and no histamine, heparin

6. Measurement of the fluorescence emission decay time-constants at 10^{-9} and 10^{-12} sec. of chlorophyll, using laser-synchronous light and the use of the photomultiplier and C^{14} or tritium labelled precursors results in the formation of "monolithic chlorophyll" in vivo or in vitro. It is proposed that these monolithic chlorophyll configurations fluoresce. A number of possible mechanisms whereby the transmission of light energy through monolithic chlorophyll may occur are discussed.

7. The presence of chlorophyll in an intact photosynthetic membrane cell which exhibits chlorophyll-mediated photosynthetic cells is discussed.

8. The primary photochemical in which reduces of carbon monoxide-mediated light energy of chlorophyll in the photosynthesis-linked cells is discussed.

9. Their measurement of the absorbance of the cytochrome of chlorophyll-mediated photosynthetic cells is illustrated.

10. The accumulation of the light-mediated formation of the cytochrome cellular cells is described. It resembles mainly of chlorophyll.

11. The accumulation of the chlorophyll and carbon dioxide of the light cellular cells is mainly mediated photosynthetic fragments is described. The inactive particles of the chlorophyll having activity and no carbonate, respiration

or 5-HT. Another fraction contained 100% of the 5-HT and histamine, 53% of the heparin, but only 28% of the mitochondrial enzyme activity.

12. The absence of monoamine oxidase and histaminase in the Dunn-Potter mastocytoma is discussed.

Fig. 1: A typical ascites Dunn-Potter mastocytoma cell showing the granules (G) which are easily distinguishable from the mitochondria (M). The nucleus (N) is in the upper left-hand corner. Vesicles of the agranular-reticulum (AR) are scattered throughout the cytoplasm. The highly active cell surface ingesting extracellur fluid is apparent. (X15,000).

Fig. 2: High magnification picture of ascites mastocytoma cell showing membrane-bound granules (G) some of which can be seen to be made up of still smaller granules and vesicles. Nucleus (N) with a double membrane and abundant agranular reticulum (AR) are apparent. (X35,000).

Fig. 3: An ascites mastocytoma cell. In the cytoplasm adjacent to the nucleus (N) are aggregates of micro-granules and microvesicles (G1, G2, G3). The vesicles in G1 are clearly enclosed within a membrane. The similarity between these micro-granules and microvesicles and those of the agranular reticulum (AR) (see also Fig. 2) is clear. In G1, G2, and G3, some of the microbodies appear to have clumped together, producing areas resembling the mature granules seen in Fig. 2. G1, G2 and G3 could be early forms of the mast cell granules, thus indicating the agranular reticulum as the origin of the granules. (X30,000).

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Fig. 1

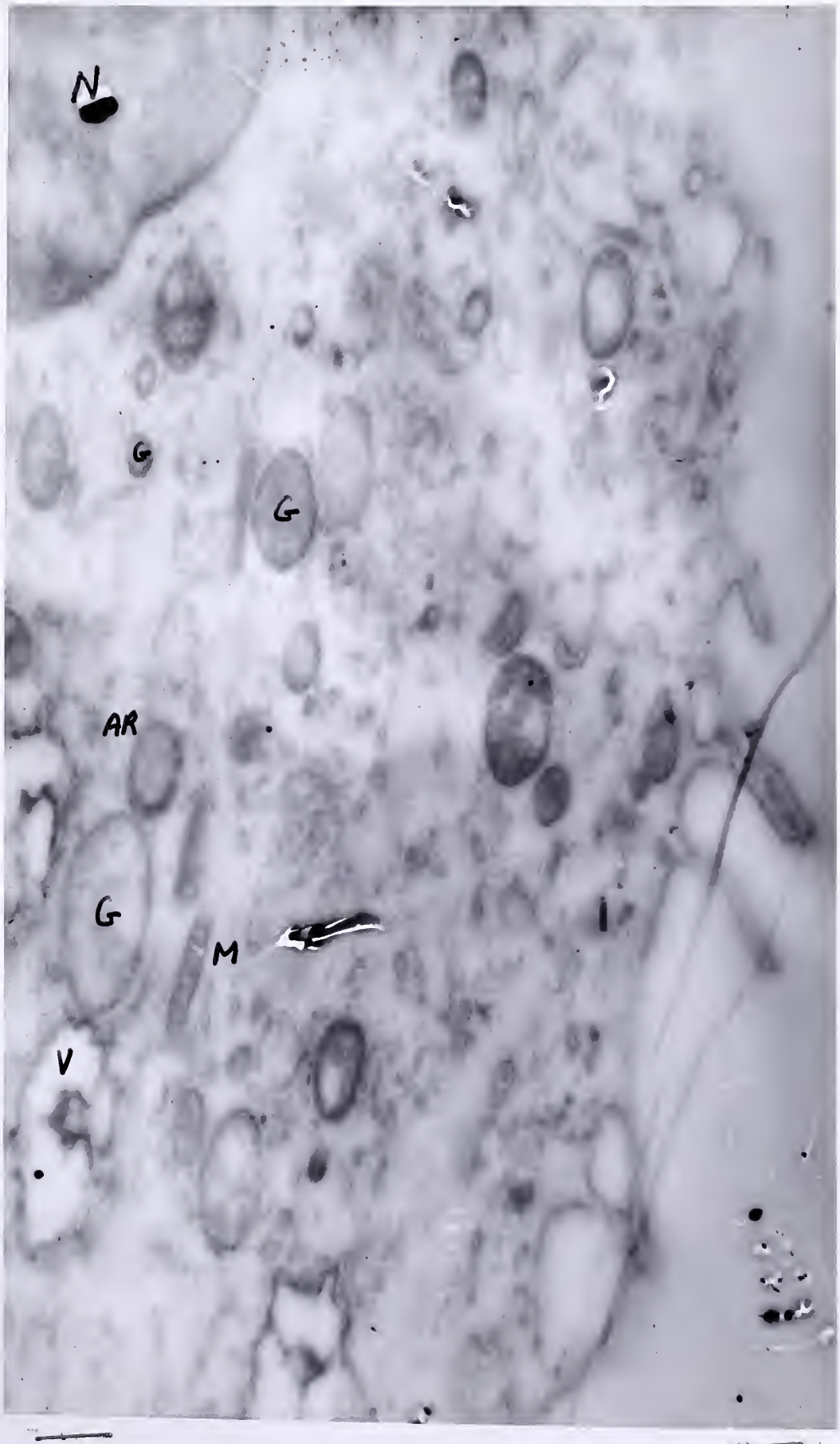


Fig. 2

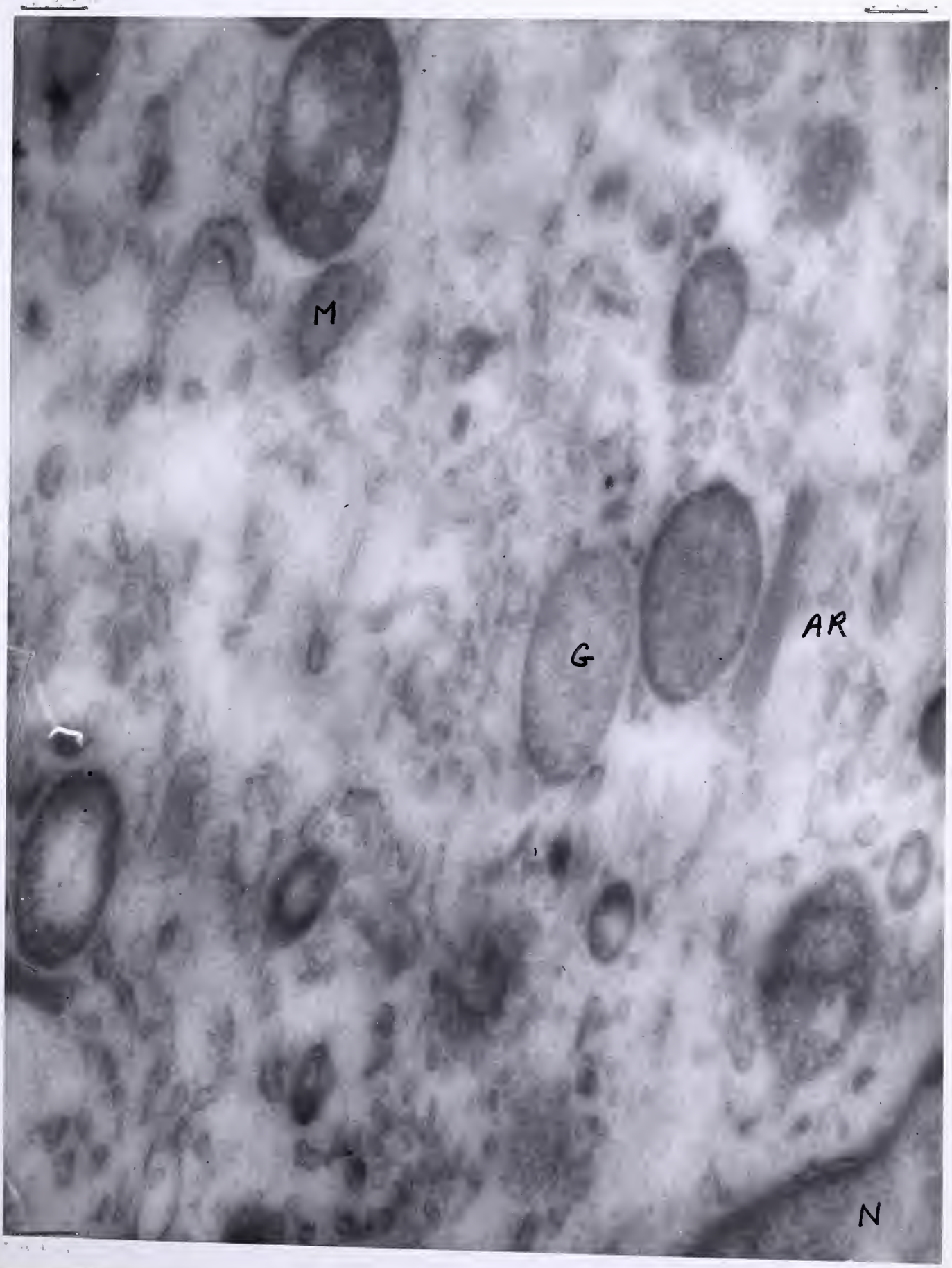
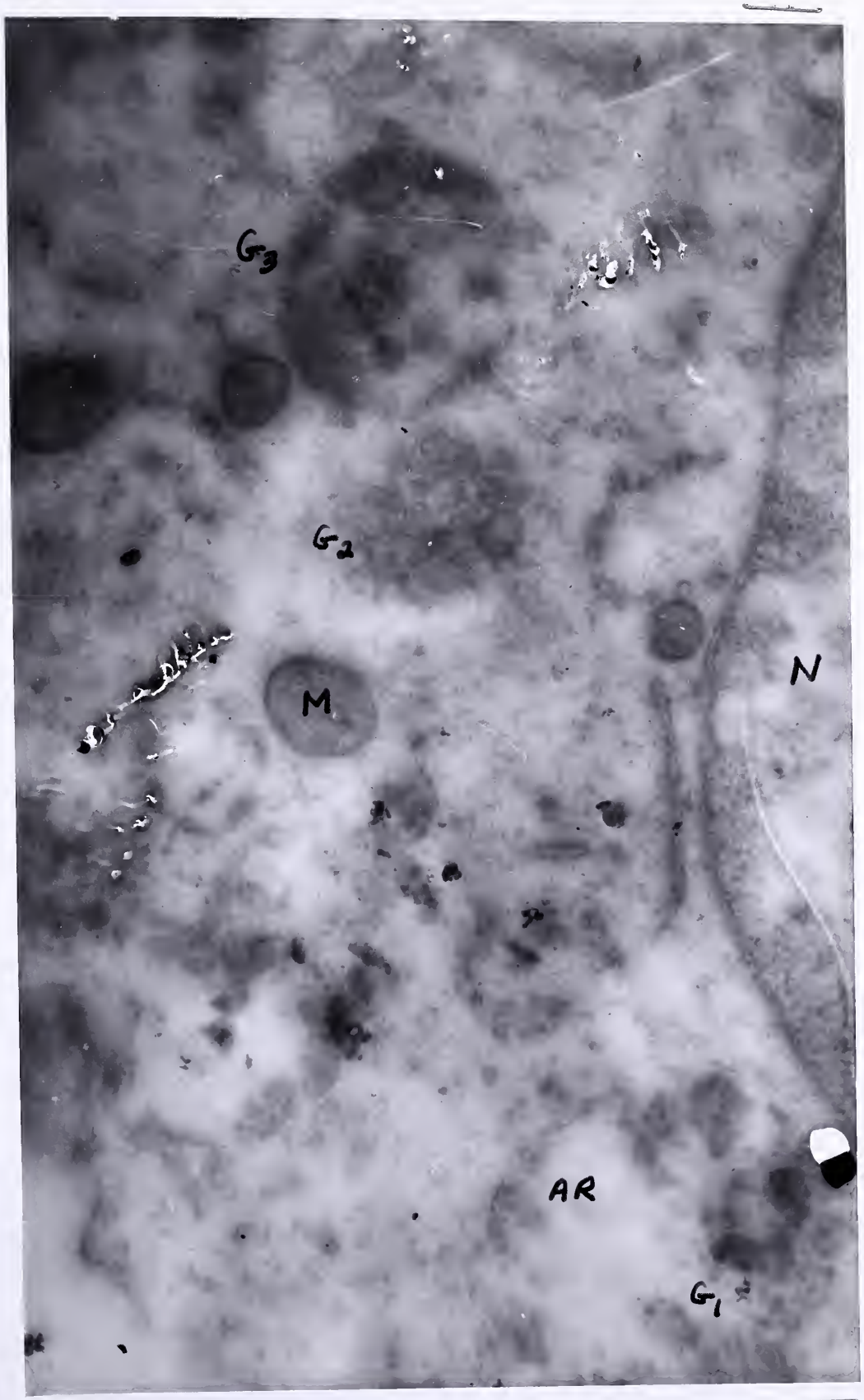


Fig. 3



Figs. 4, 5: Two typical tissue culture mastocytoma cells. Nucleus (N) with double membrane (NM) is evident as are a great many mitochondria (M). A few cytoplasmic inclusions seem to lack cristae and could be either degenerated mitochondria or granules (A). The border (B) between two adjacent cells is evident in Fig. 5, as is a large lipid body (Li). (Fig. 4 X18,000; Fig. 5, X16,000).

Fig. 6: One of the few tissue culture cells in which the endoplasmic reticulum (ER) was seen.

Fig. 7: High power picture of tissue culture mast cell with cytoplasm filled with numerous mitochondria (M), agranular reticulum (AR), and two lipid bodies (Li). Note absence of granules. (X43,000).

Fig. 8: Several tissue culture cells closely adherent to one another with small, membrane-bound granules (G) scattered through the cytoplasm and easily distinguishable from the mitochondria (M).

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Fig. 4

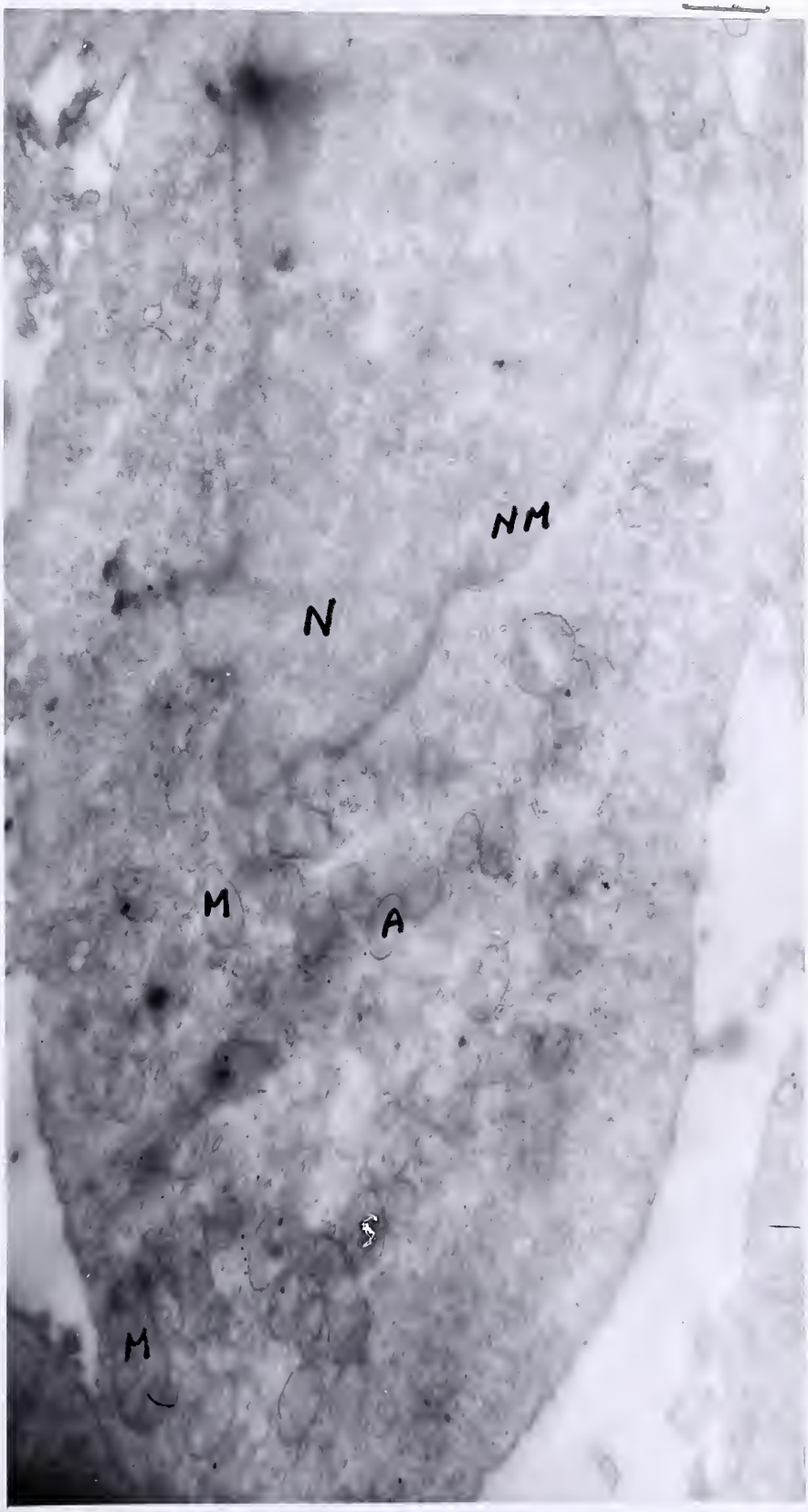


Fig. 5

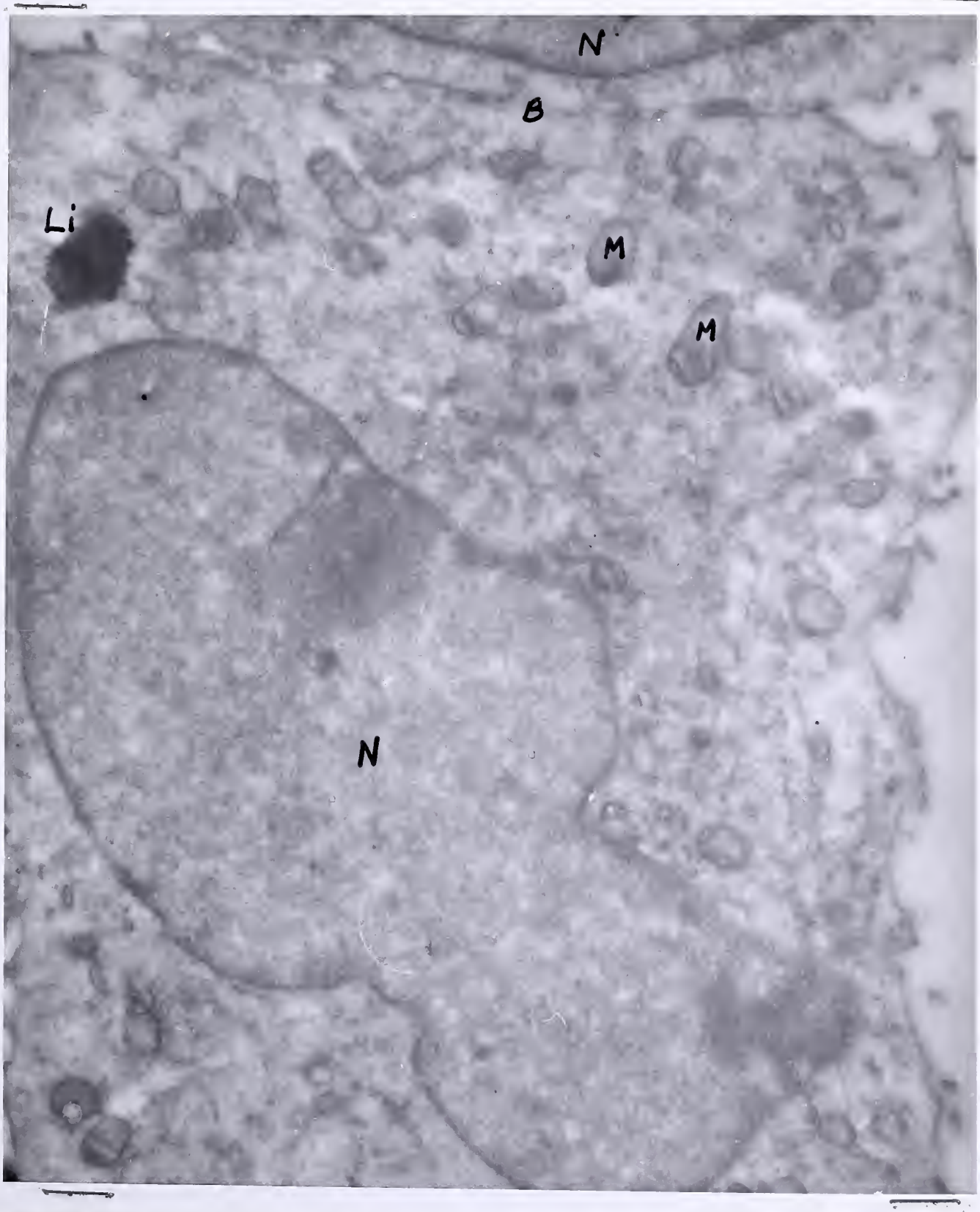
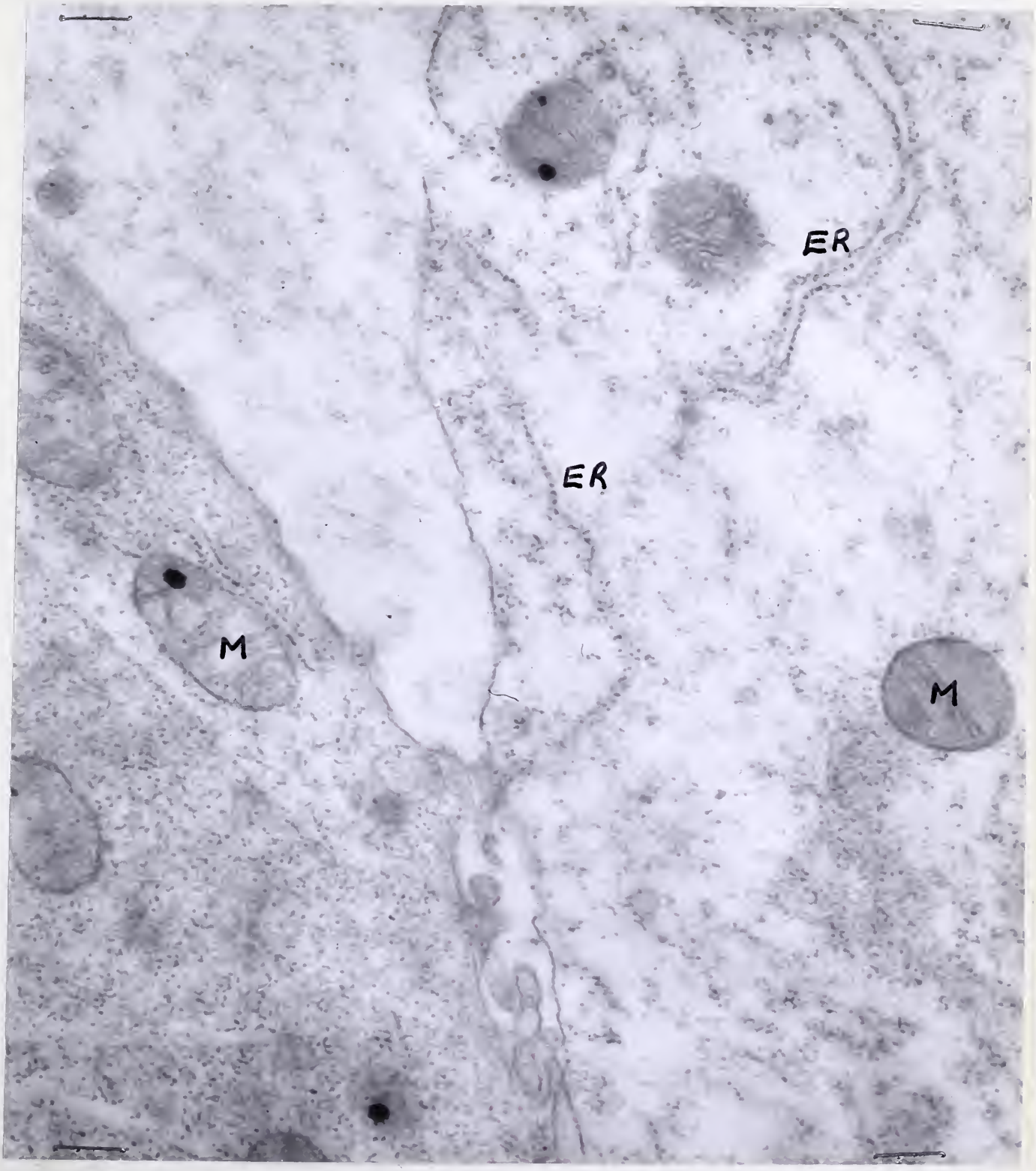


Fig. 6



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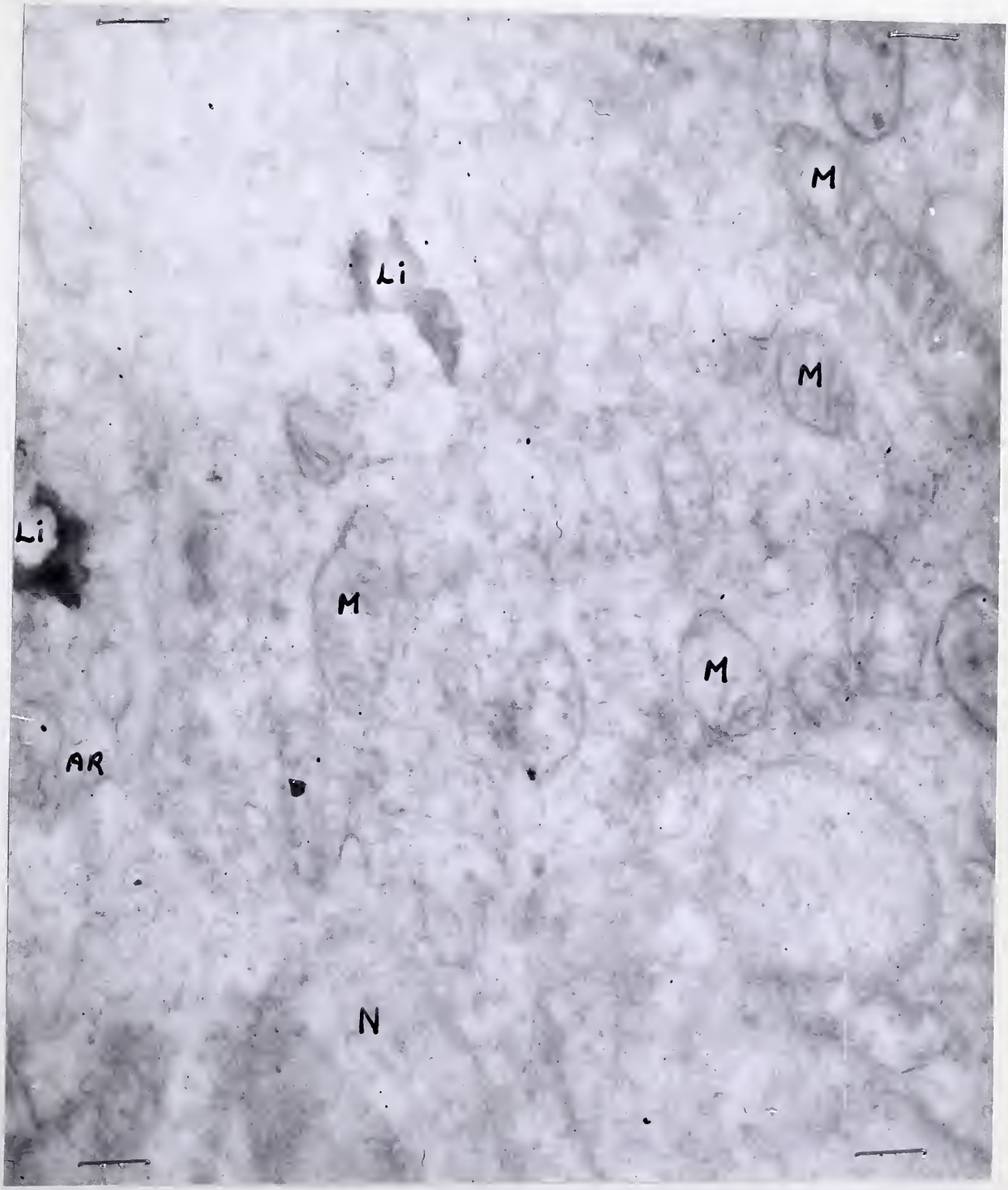

Fig. 8

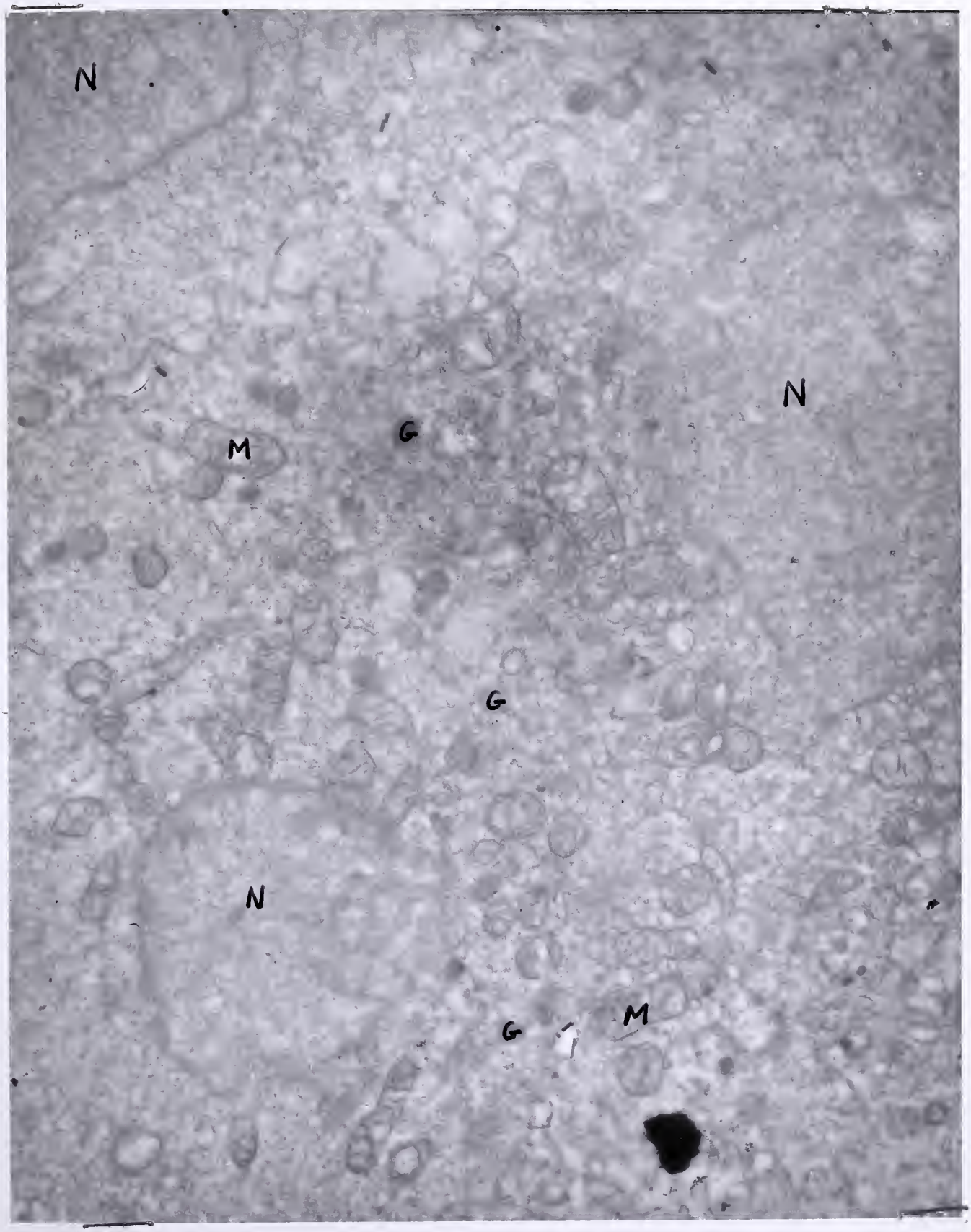

Fig. 9: A tissue culture cell with many very bizarre inclusions (L). They resemble somewhat the vacuolated dense bodies (probably lysosomes) seen after reserpine treatment of these cells. (X13,000).

Fig. 10: A tissue culture mastocytoma cell treated with reserpine. Note the granules (G), some of which are within the cell and surrounded by a clear area of cytoplasm, and other granules which have been or being extruded at the cell surface in the upper right-hand corner. No nucleus is present but this may be due to the plane of sectioning. Mitochondria (M) and lipid bodies (L) are present. Note the difference between this cell and the other tissue culture cells (Figs. 4-7) and the similarity to a normal mouse mast cell (Fig. 12). (X12,000).

Fig. 11: A high power shot of the same cell seen in Fig. 10. The granules (G) are seen more clearly as well as the extrusion of granules at the cell surface. (X30,000).

Fig. 12: A normal mouse mast cell. Note how few granules are present and the clear area around "each granules." Nucleus (N) and mitochondria are apparent. Compare with Fig. 10 (X7,000).

Fig. 9

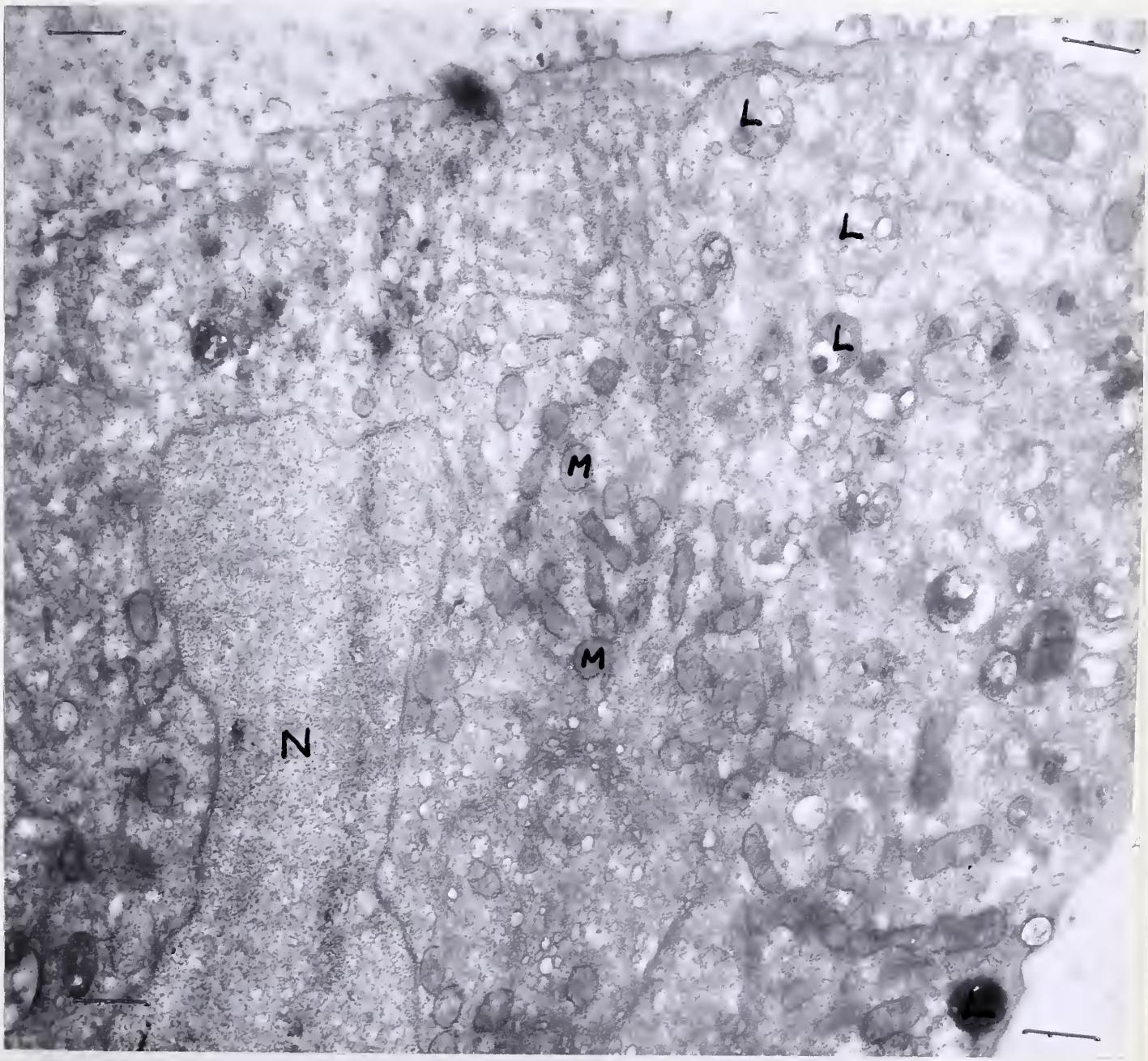

Fig. 10

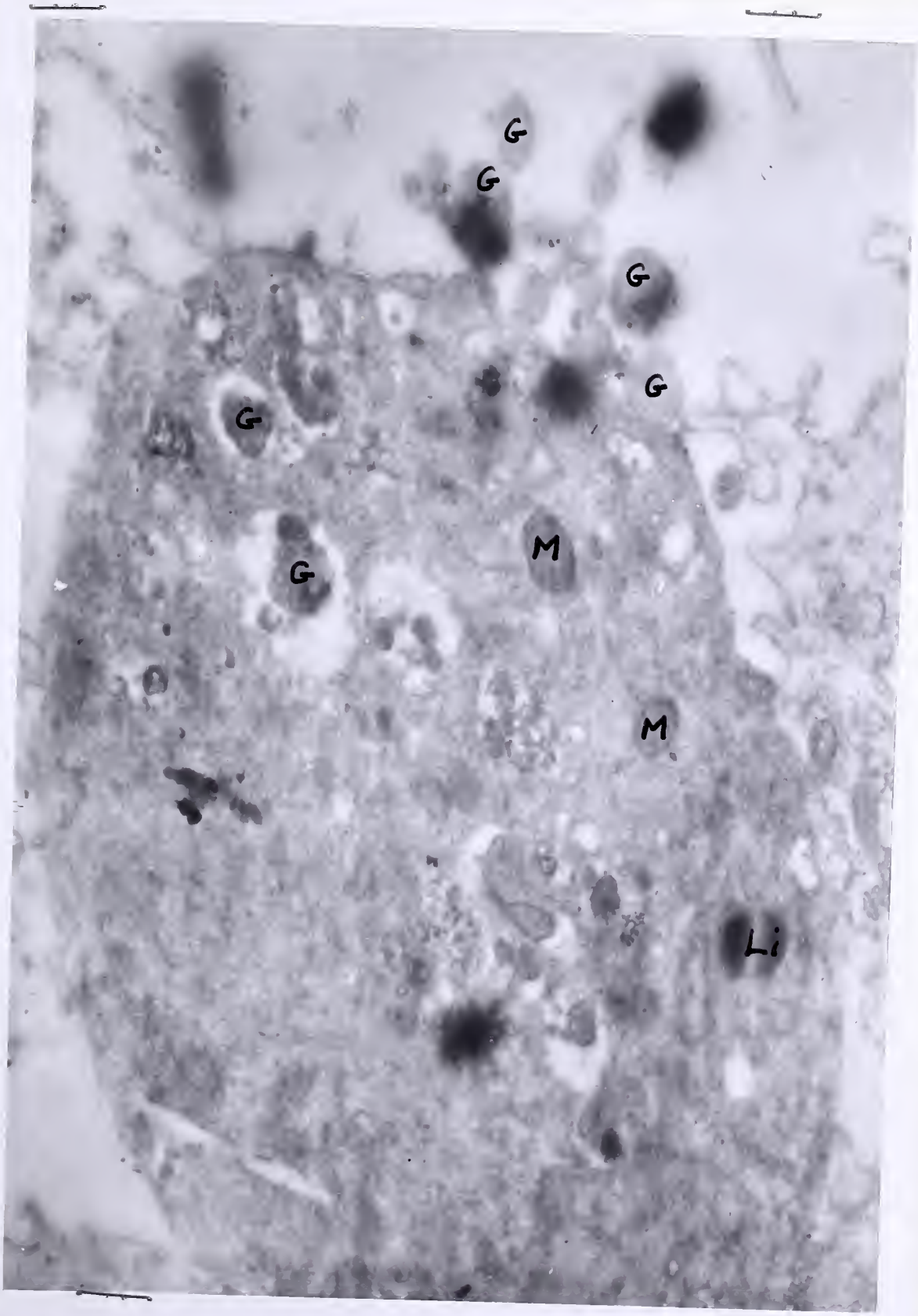

Fig. 11

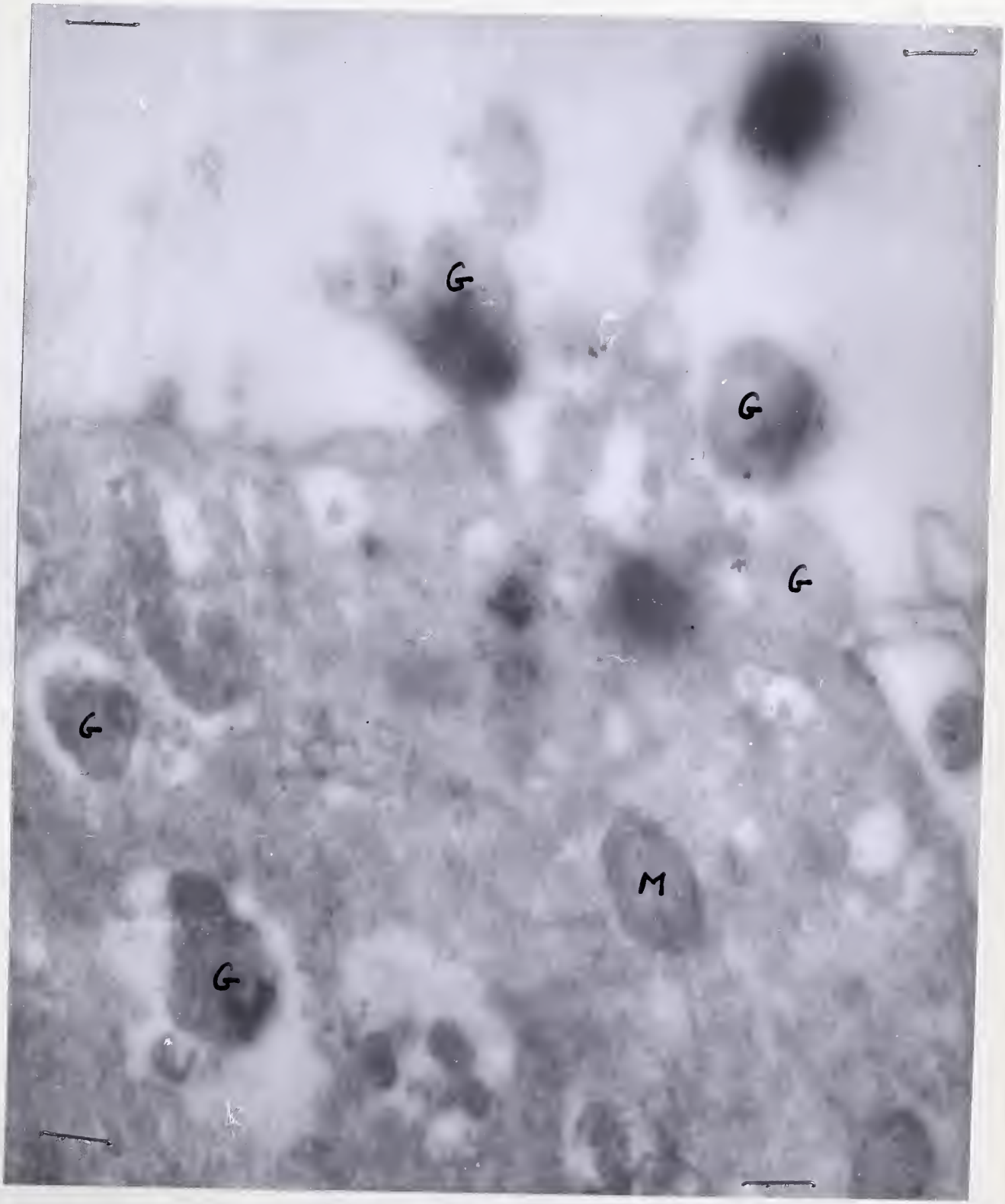

Fig. 12

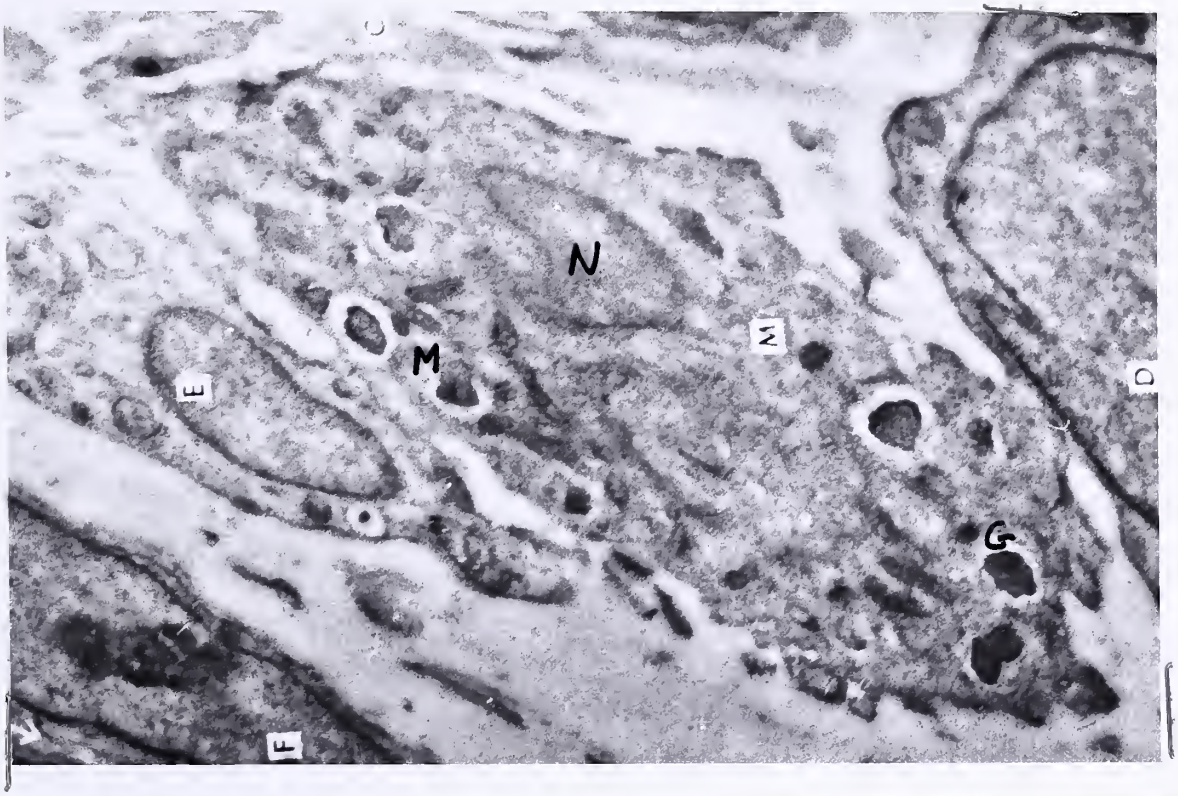

Fig. 13: Portion of an unusual mastocytoma cell, much larger than any other cell, with four nuclei (N). Mitochondria (M), granules (G), and lipid bodies (Li) are seen. Many of the granules are made up of microgranules (Gi, G2, G3). Note the similarity between these granules and those of the ascites cell. (Figs. 2, 3). Small granules (SG) are scattered throughout the cytoplasm. (X12,000).

Fig. 14: Another region of the same cell seen in Fig. 13. G1, G2 and G3 are granules whose fine structure is clearly evident. G4 is a mature granule. Numerous small granules, mitochondria, and agranular reticulum are scattered throughout the cytoplasm. (X12,000).

Fig. 14: An unusual tissue culture cell with large clear areas in the cytoplasm and two large lipid inclusions (Li). The clear areas (G) probably represent granules. Mitochondria (M) are located between the granules. (X18,000).

Fig. 13

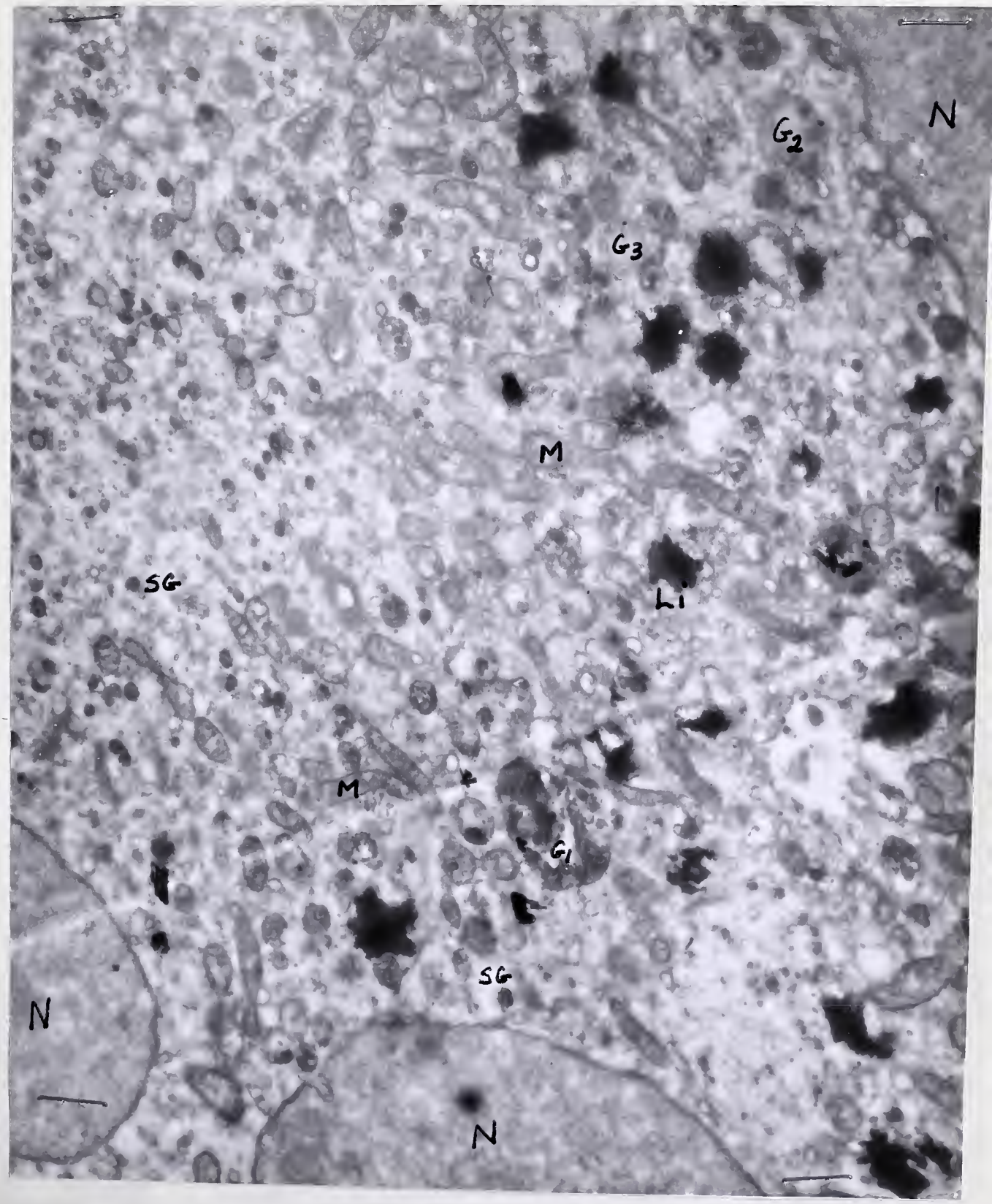

Fig. 14

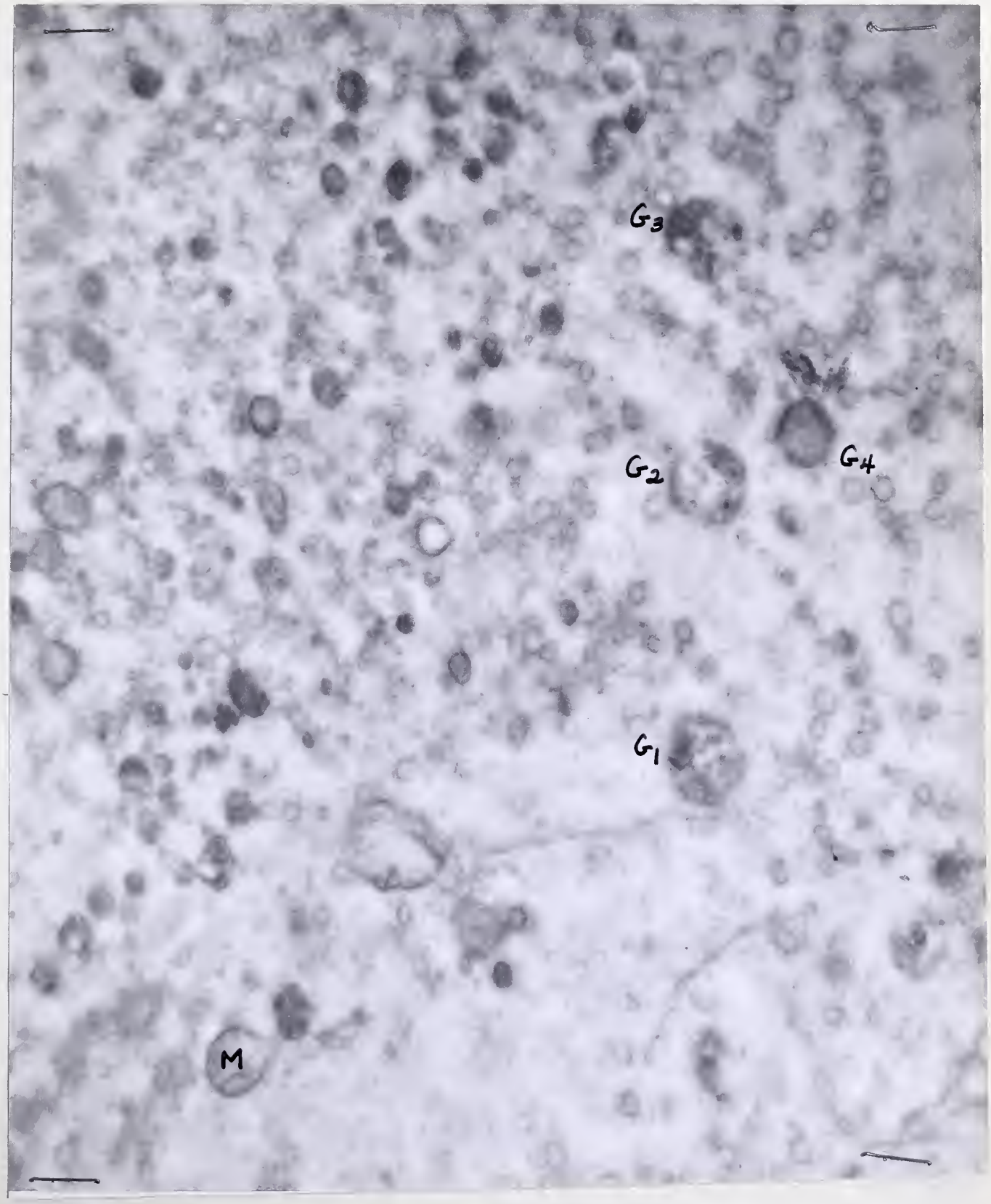

Fig. 15

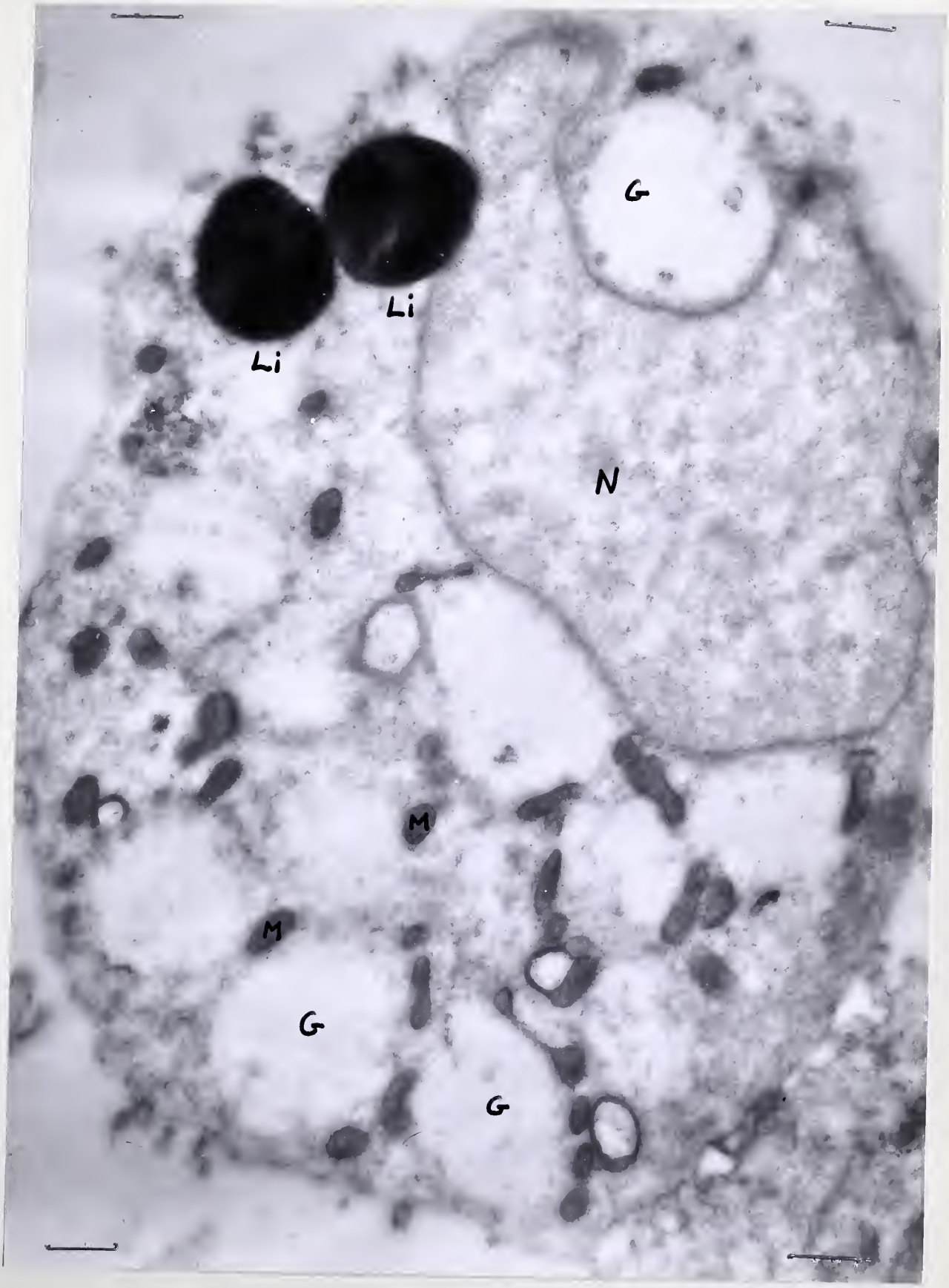

Fig. 16: Portion of a tissue culture cell treated with reserpine at 10^{-9}M for 24 hours. Numerous vacuolated dense bodies are seen (L). Large vacuoles (V) containing smooth membranes and osmophilic matter are evident. The vacuolated dense bodies could be formed from the material in these vacuoles. The cell membrane is intact. (X15,000).

Fig. 17: Another cell filled with vacuolated dense bodies (L). The relationship of these to mitocholdria (M) is evident at the areas where the mitochondria are marked. The mitochondria appear to be undergoing dissolution in connection with the formation or activity of the vacuolated dense bodies. (X15,000).

Fig. 18: Two tissue culture cells treated with reserpine at 10^{-9}M for 24 hours. The arrow indicates a granule which is being extruded from or budded from one of the cells. A granule (G) is apparent in the second cell. (X20,000).

Fig. 19: Tissue culture cell treated with reserpine at 10^{-9}M for 24 hours. Membrane-bound amorphous cytoplasmic areas (I-IV) at the cell surface and deeper within the cytoplasm may have been produced by discharge of cytoplasmic constituents at the cell surface. Gaps in plasma membrane are evident in areas I, IV, V. (X15,000).

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Fig. 16

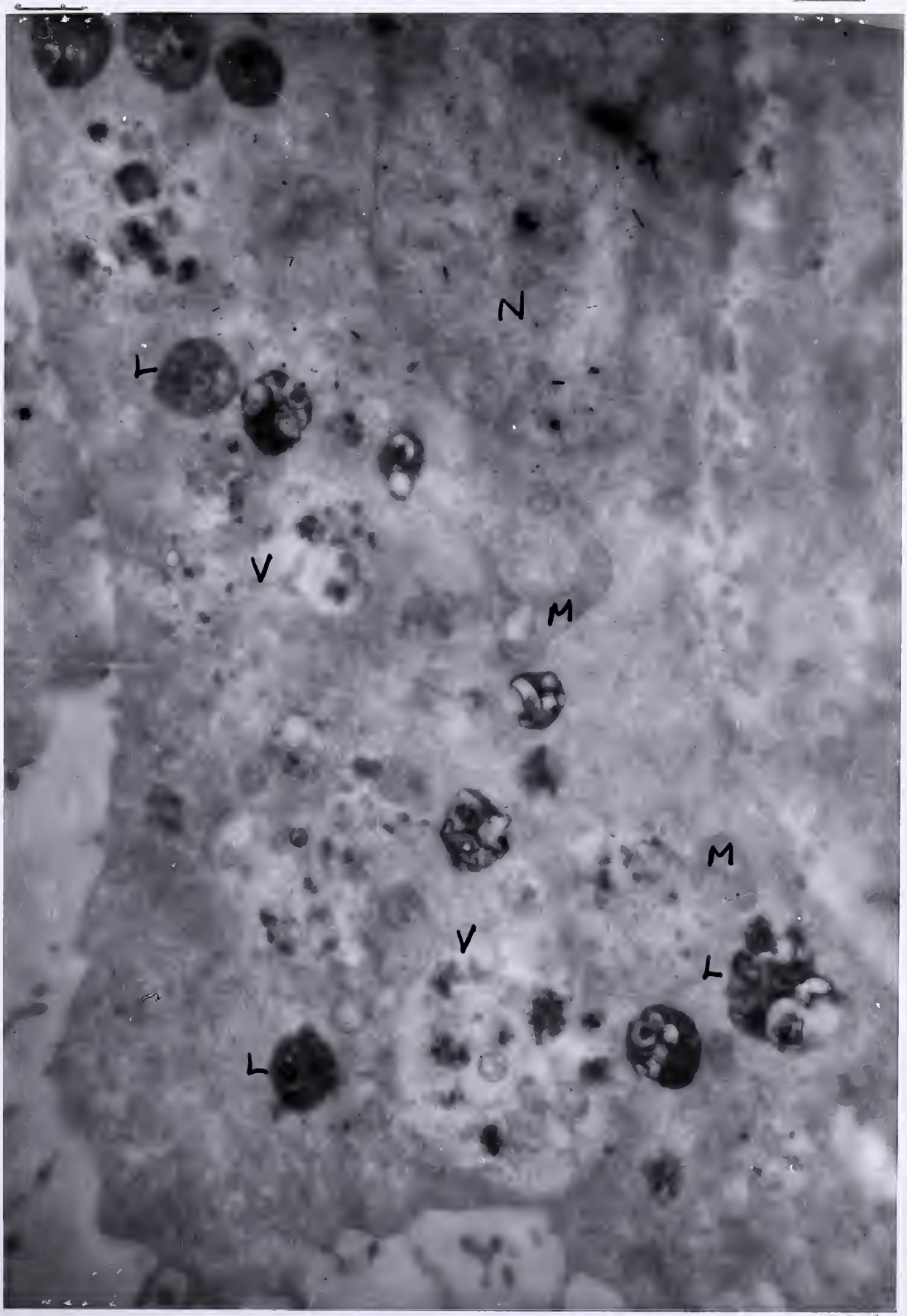


Fig. 17

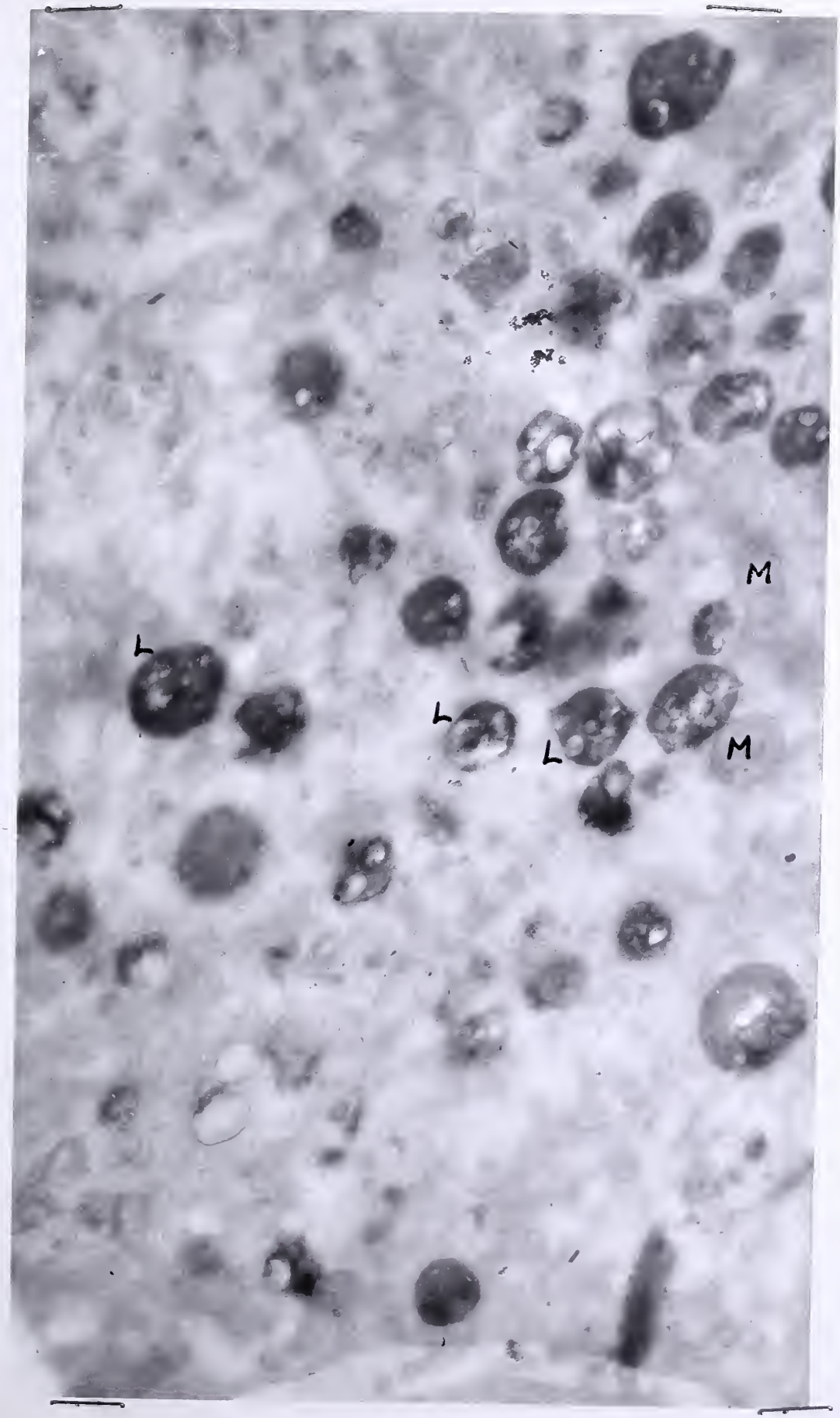


Fig. 18

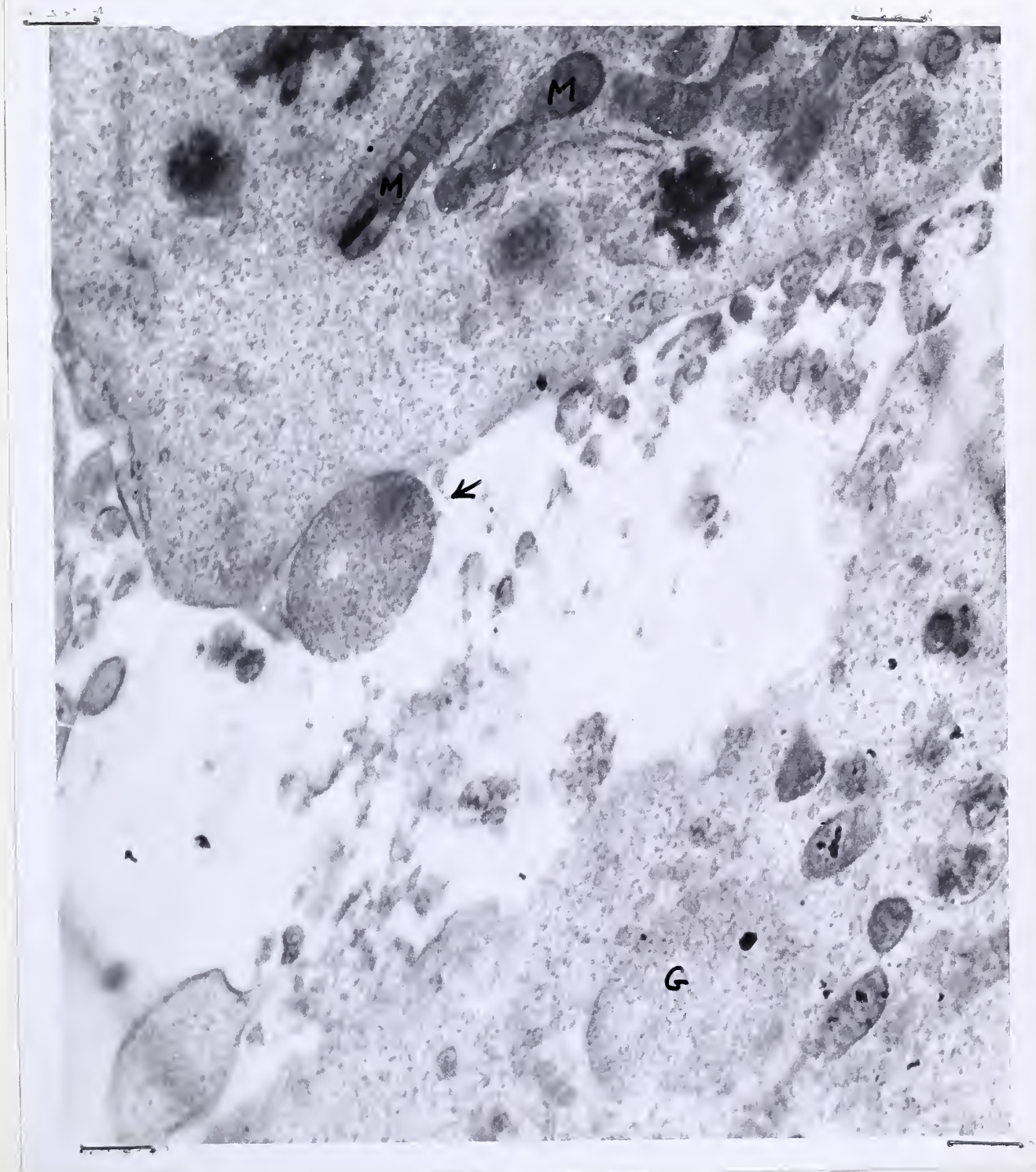
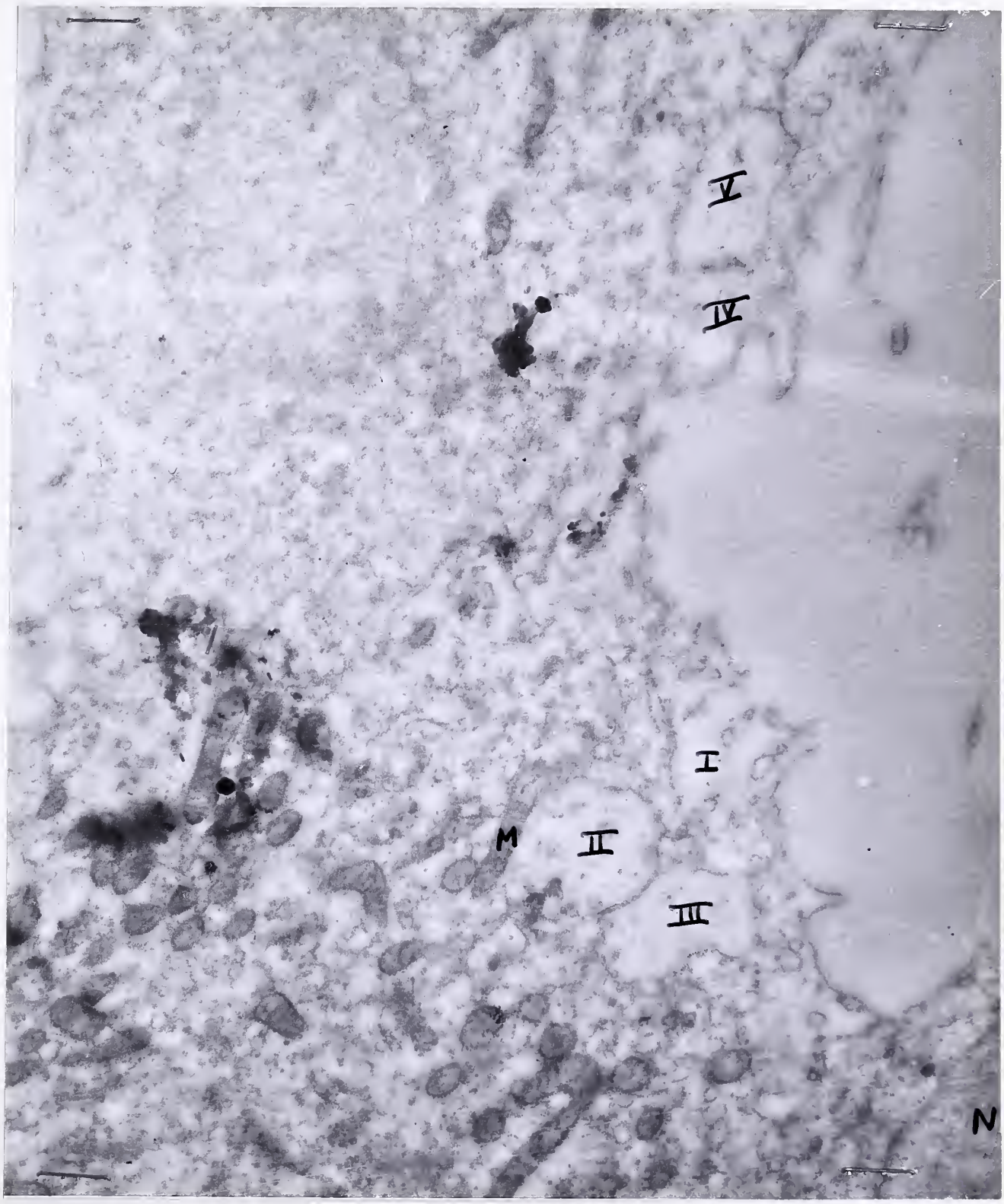


Fig. 19



Figs. 20-22: Tissue culture cells treated with chlorpromazine at 10^{-5}M for 12 hours. Practically all the mitochondria present have grossly distorted internal structures. Note that the outer membrane of some of the mitochondria is partially absent as well. Some of the mitochondrial membranes have a whirled configuration. (Fig. 20, X20,000; Fig. 21, X25,000; Fig. 22, X15,000).

Fig. 23: Normal mitochondria of tissue culture cells for purposes of comparison with cells treated with chlorpromazine. Note two parallel membranes of the cristae which are perpendicular to the outer mitochondrial membrane. See also Figs. 4-8. (X30,000).

Fig. 24: The large granule fraction from the ascites cells consisting mainly of battered mitochondria. A few granules are evident elsewhere amongst the degenerated membranes (X20,000).

Table 20-1: Cause of tumors with energy zero chlorophyll as low as 10^{-3} for it to be. Functionality and the radiomimetic cannot have greatly affected Jackson's experiment. Note that the a-ray decrease of some of the mitochondria in yeastlike growth as well. Some of the mitochondrial genes have a normal mutation. (Fig. 20, 250,000; Fig. 21, 200,000; Fig. 22, 312,000).

Fig. 23: Normal mitochondria in Pinus ponderosa cells for purposes of comparison with cells exposed with chlorophyll. Note the partial membrane of the cytoplasm which are perpendicular to the cross sectional membrane. See also Figs. 1-9, (240,000).

Table 24: The large amount transition from the radiation cells cytoplasmic mitochondria. Ray chemicals are evident. Magnifying because its homogenous membrane (250,000).

Fig. 20

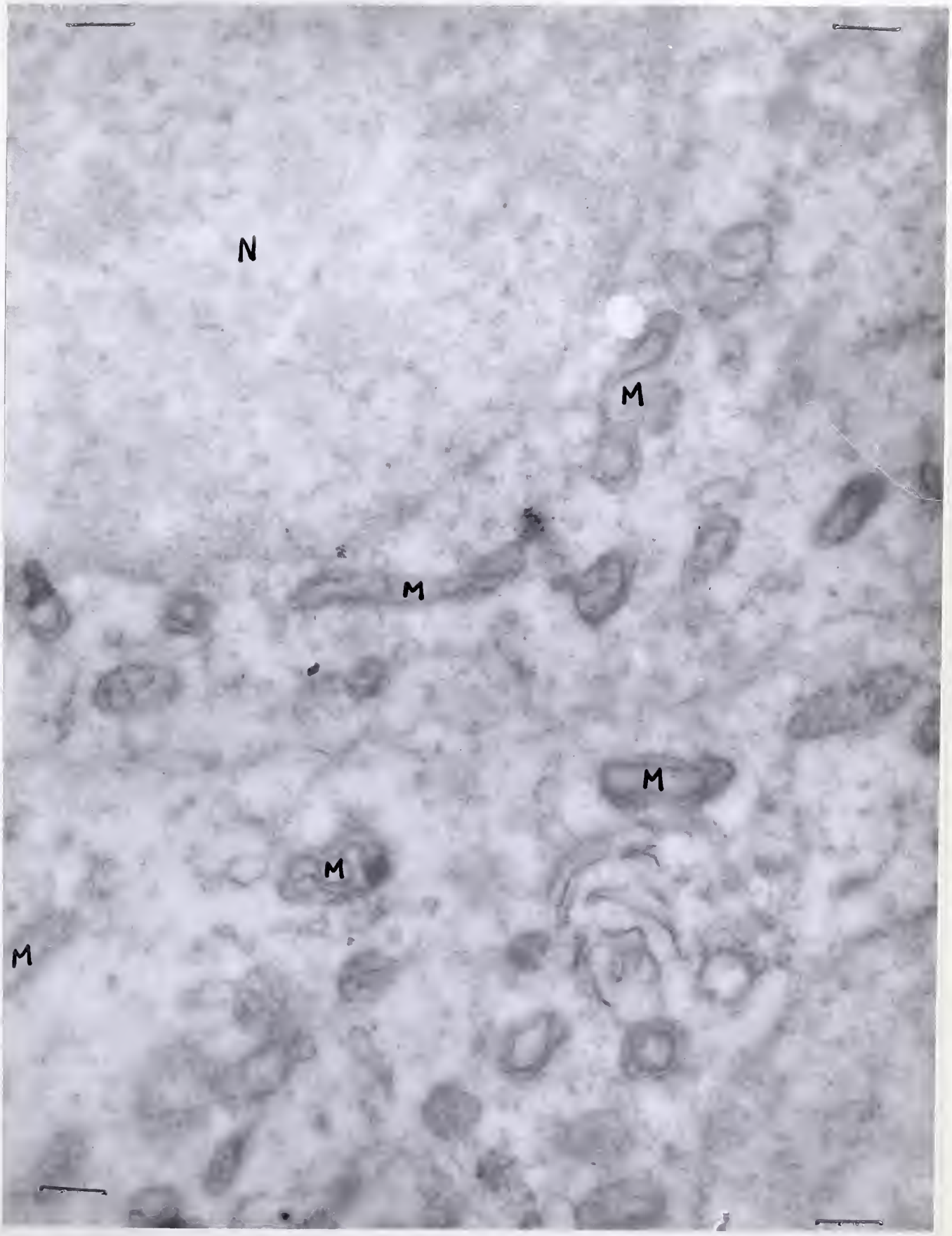


Fig. 21

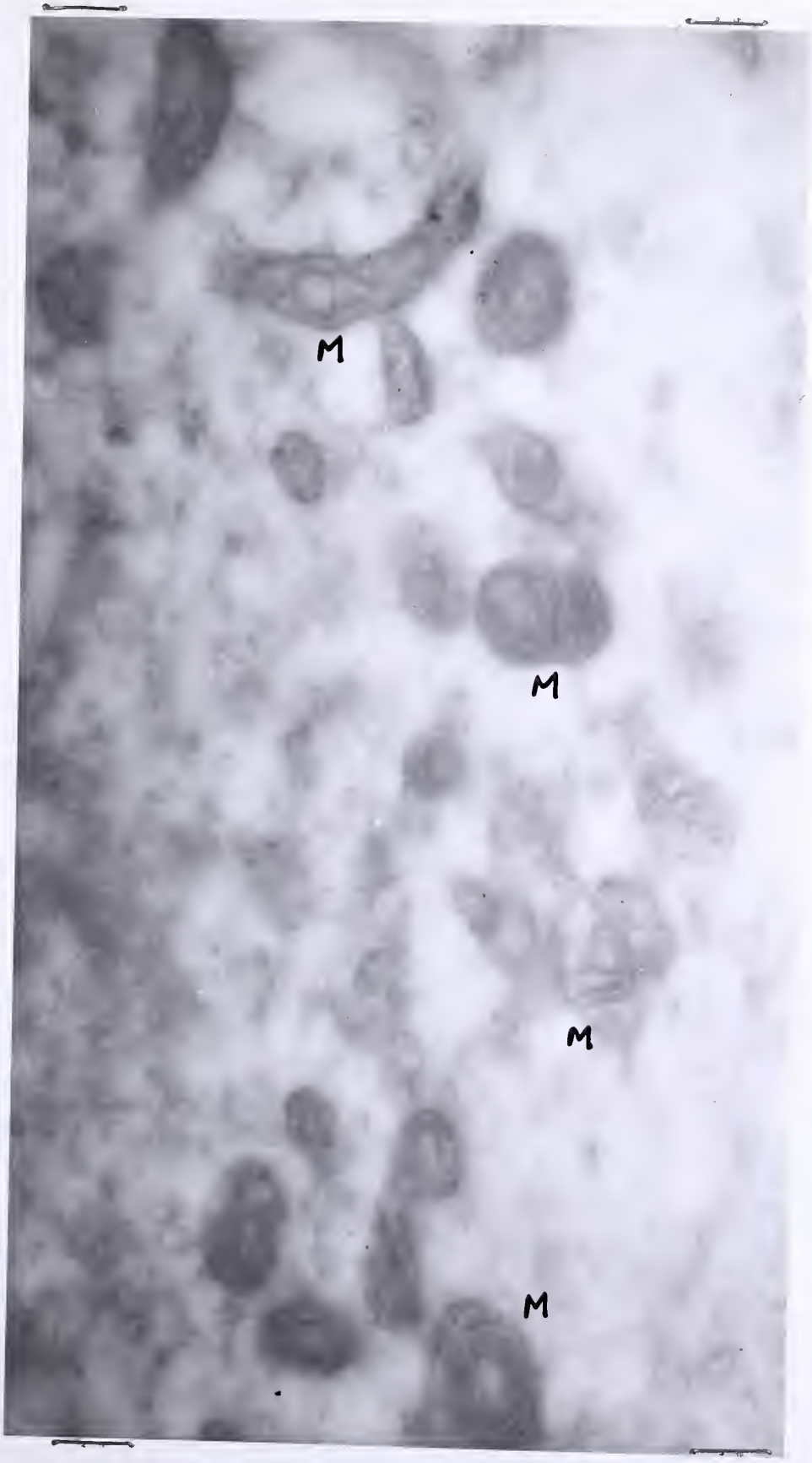


Fig. 22

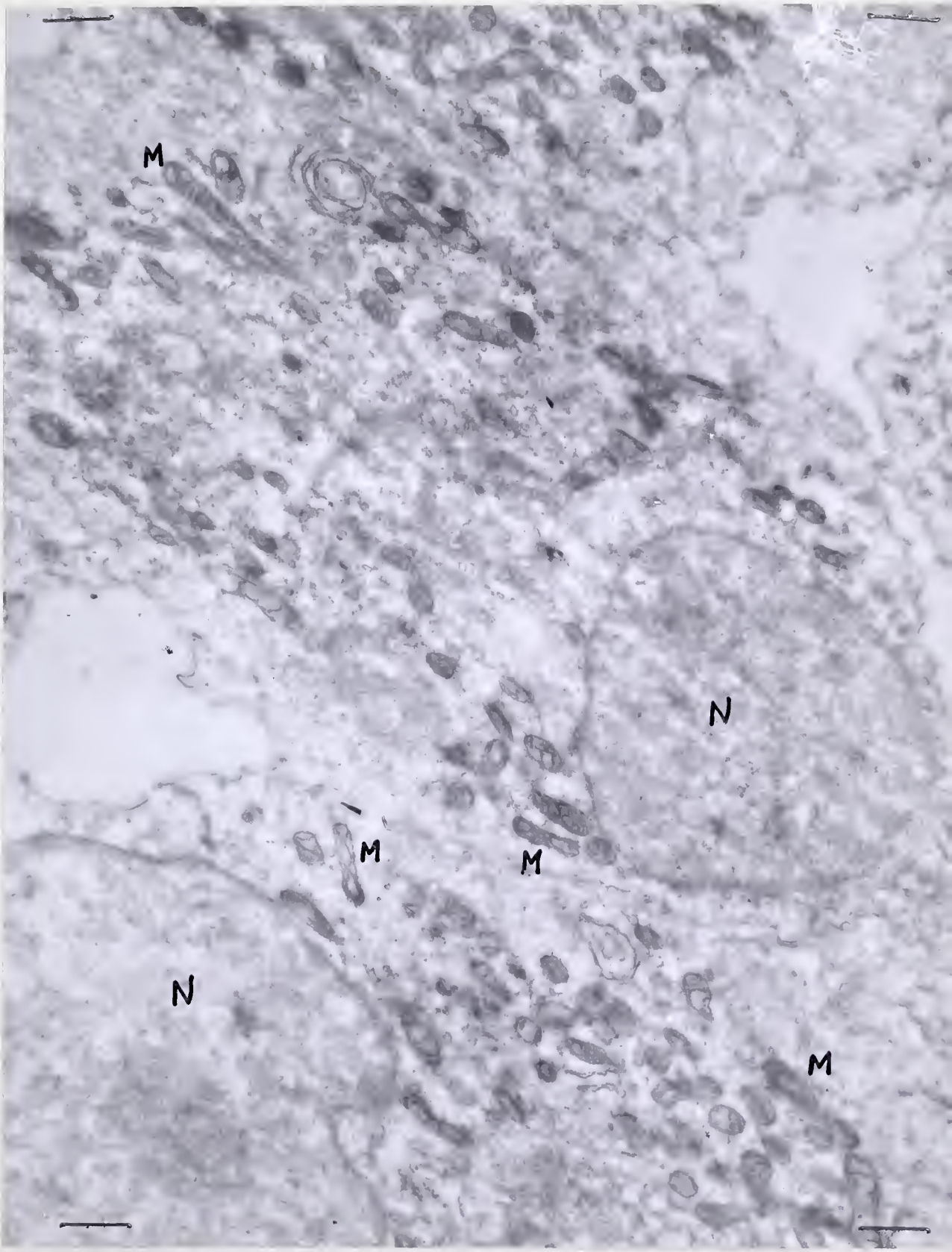


Fig. 23

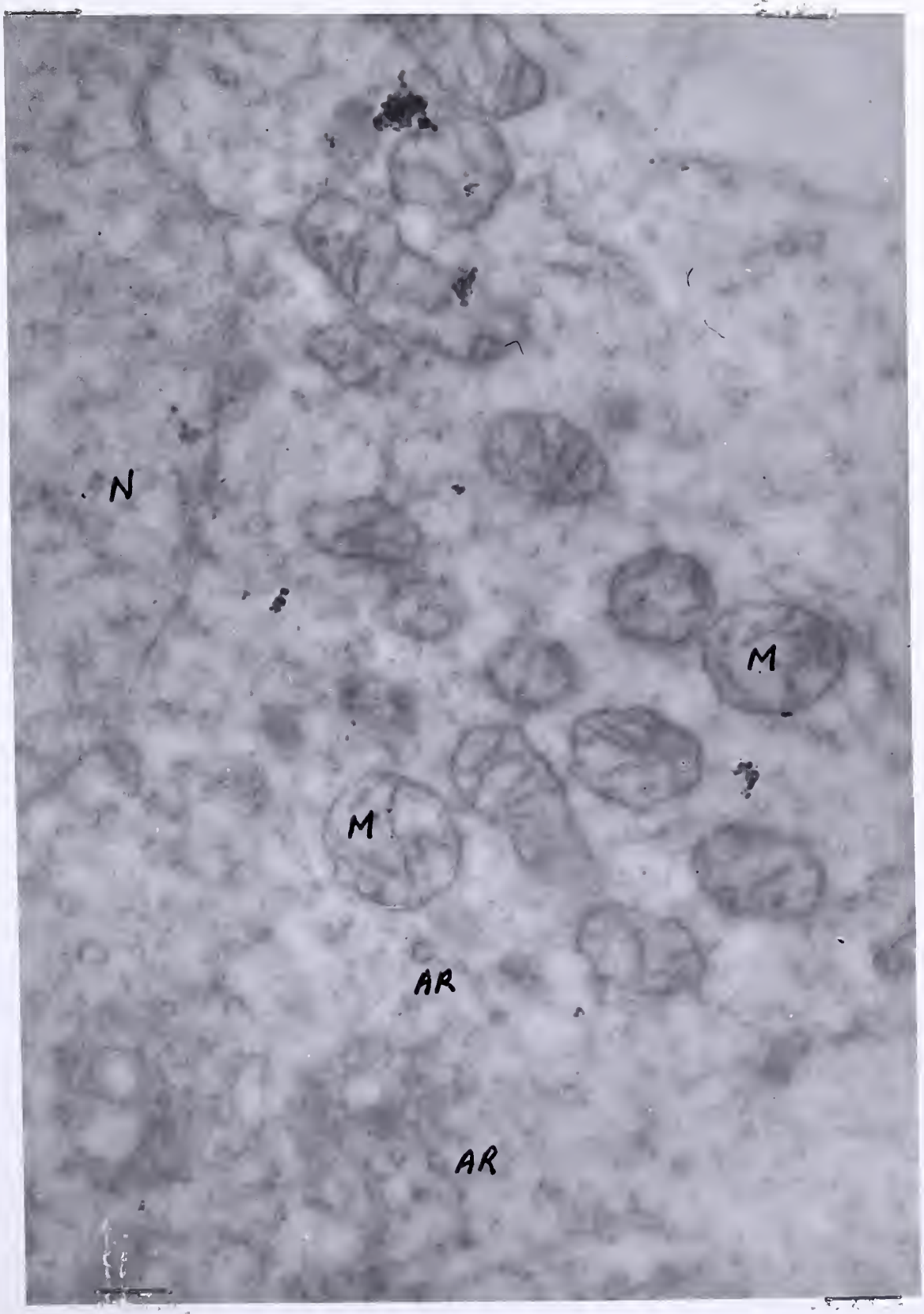
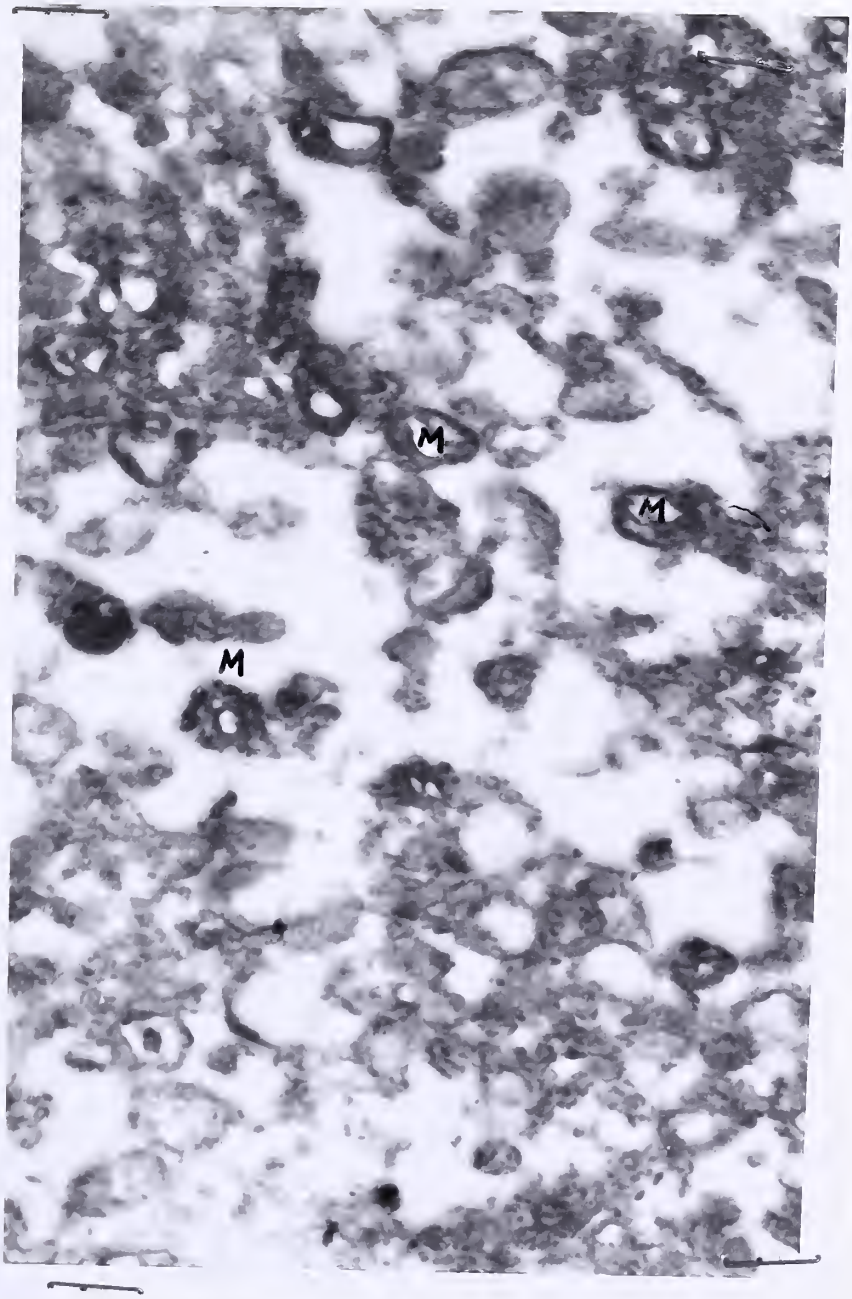
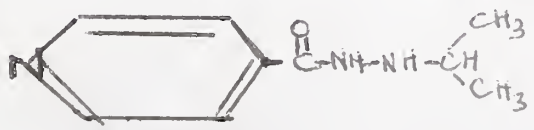


Fig. 24



INTRODUCTION

The first evidence for the oxidative deamination of amines by living tissues was the discovery in 1928 of a liver enzyme which oxidized tyramine.[145] Further studies in the 1930's on the enzymes which metabolize amines through oxidative deamination led to the concept that were was a single enzyme which degraded diverse monoamines such as tyramine, the catecholamines, and aliphatic amines, and another which oxidized diamines such as putrescine, the former being named monoamine oxidase (MAO), and the latter, diamine oxidase.[146-148] It was the idea that MAO could be related to central nervous system function that made it such an interesting enzyme to investigate. In 1940, Mann and Quastel observed that amphetamine inhibited MAO *in vitro* and suggested that the stimulant effect of amphetamine might be due to inhibition of MAO *in vivo* by blocking the formation of certain amine degradative products which they believed had a depressant effect.[149] However, no inhibition could be demonstrated *in vivo* after administration of sufficient amphetamine to produce stimulation. The next stimulus to work in this area was the finding by Zeller *et al.*,[150] that isoniazid and iproniazid, "hydrazines" that were active as



Iproniazid

anti-tuberculous agents and which clinically had the "side-effect" of being central stimulants,[151] were potent inhibitors

of MAO *in vitro* and *in vivo*. Subsequent studies demonstrated that reserpine pretreatment produced marked excitation rather than depression when given to animals pretreated with iproniazid and that it no longer possessed its capacity to lower brain 5-HT and catecholamine levels in these animals.[152,153] These observations led to the testing of iproniazid as an antidepressant.[154] The success of this clinical trial and the assumption that the mode of action of iproniazid was the inhibition of MAO attracted a great many investigators to this area. Since this finding, hundreds of basic and clinical studies of MAO and its inhibitors have been carried out in an effort to explore the relationship of this enzyme, its substrates, and its inhibitors, with mental illness, affective states such as depression and stimulation, angina pectoris and many other conditions.

A possible explanation of the anti-depressant action of the monoamine oxidase inhibitors (MAOI) was soon forthcoming with the finding that the MAOI caused the accumulation of brain 5-HT and catecholamines, presumably by preventing their breakdown.[155-157] The accumulation of these amines which are believed to function as neurotransmitters,[158] was, and is believed by some to exert an anti-depressant effect per se. It should be mentioned that rigidly controlled clinical studies on the efficacy of the MAOI as anti-depressants have not always yielded unequivocal results; and because of the toxicity of these compounds, they are now used infrequently as compared to imipramine (Tofranil) which is not a MAOI and which most studies

have shown to be a more effective and less toxic mood elevator).[159]

The multiplicity of effects of the hydrazines on other enzymes and on various parameters of amine biology other than just accumulation of amines, have greatly complicated the explanation of the anti-depressant action of these compounds. The hydrazines have been shown to inhibit diphosphopyridine nucleotidase,[160] spermine oxidase,[161] guanidine deaminase,[162] succinic dehydrogenase,[163] histaminase,[164] and even the decarboxylase which brings about amine synthesis from precursor amino acids (by interfering with coenzyme of the decarboxylase, pyridoxal - 5' - phosphate).[165, 166] The MAOI potentiates the action of various pharmacologic agents,[167] including the barbiturates.[168] The hydrazines have been shown to inhibit the release by reserpine of 5-HT from rat brain,[169] as well as inhibiting the release of NE from various storage sites.[170] Schanberg and Giarman have shown that phenylisopropyl-hydrazine produces an increase in the proportion of amines which are "bound" (within granules) rather than free (in the cytoplasm). Iproniazid increases the amount of both forms of the amine proportionately and does not change its distribution.[171] Dubnich et al.[172] have shown that 5-HT levels in rat brain continue to increase in response to doses of phenethylhydrazine which exceeded the dose required for the complete inhibition of MAO. This was though to be due to the blockade of amine release. Others have shown that the various MAOI increase the amount of rat brain adenonsine triphosphate and

suggest this augmented energy supply is the reason for the anti-depressant effect.[173] Gey and Pletscher have shown that MAOI can produce increased blood lactic acid levels.[174] There are many studies which demonstrate peripheral effects of the MAOI such as sympathomimetic effects,[175] ganglionic blockade[176, 177] (but see also[77]) and adrenergic blocking action.[172a]

Despite the embarassing wealth of findings, the most reasonable explanation of the mood-elevating effect of the hydrazines, is the inhibition of MAO and blockade of amine-release, leading to the accumulation of brain 5-HT and possibly other amines. The main evidence in favor of this choice is that many compounds which inhibit MAO seem to have an anti-depressant effect irrespective of their structure, although their is no correlation between *in vitro* and *in vivo* studies. (Usdin and Usdin examined an exhaustive series of psychotropic compounds for MAO inhibition *in vitro*. They found some but not all hallucinogens, tranquilizers and anti-depressants were MAOI *in vitro* and concluded: "No obvious relationship between psychotropic action and MAO inhibition can be discerned."[173a] As previously mentioned, however, studies *in vitro* are of limited value in this field because of a host of factors, such as metabolism of the agent *in vivo* and regional specialization of the brain with regard to such properties as biochemistry and permeability barriers). Additional evidence for the relationship between elevated 5-HT levels and anti-depression is that other modes of elevating the brain amines such as administer-

ing the precursors of the cathecholamines, dihydroxyphenylalanine, or the precursor of 5-HT, 5 hydroxytryptophan, are associated with central stimulation.[174a,175a] (Bonnycastle *et al.* have shown that a variety of central *depressant* drugs, including sedatives, hypnotics, analgesics, fixed and volatile anesthetics, produced a significant elevation of rat brain 5-HT. His data suggested that the 5-HT elevation was secondary to the central nervous system depression, not the cause of it).[176a] Shore[177a] noted a temporal relationship between the pharmacological effects of iproniazid and an increase in the levels of brain 5-HT and NE. The many studies relating amines to behavioral changes induced by other drugs such as reserpine are also in favor of this hypothesis. Indeed, the knowledge that iproniazid, an antidepressant, raised 5-HT levels in the brain, while reserpine, a tranquilizer, lowered 5-HT in brain, stimulated great interest in the study of brain biochemistry and pharmacology.

To compound the interest in the MAOI, studies show their efficacy in relieving angina pectoris[178, 179] their hypotensive effect,[180] their anti-convulsant effect,[181] their anti-inflammatory effect,[182,183] an analgesic effect,[184] and even an anti-fertility action.[185] They are clearly a fascinating group of compounds.

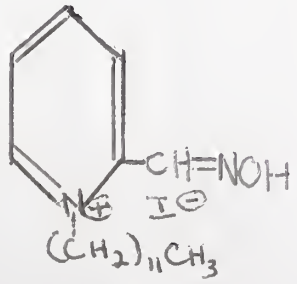
In most cases, only the hydrazines have been studied in these experiments. Whether or not the many other classes of compounds which inhibit MAO and which are not hydrazines

possess these properties as well, remains to be investigated. Among the other classes of inhibitors of MAO which have been reported, are agents which combine with sulfhydryl groups,[186] aliphatic alcohols,[187] chlorpromazine,[188] the harmala alkaloids,[189] p-tolyl ether of choline,[190] trans-2-phenylcyclopropylamine (tranylcypromine),[191] α-methyl-and α-ethyltryptamine (etryptamine),[192] N-benzyl-N-methyl-2propynylamine (pargyline),[193] N-methyl-α-methyltryptamine,[194] and others. (The work to be described here on quaternary nitrogen compounds as inhibitors of MAO was begun when only the hydrazines were established as MAOI and enthusiasm for MAOI as anti-depressants was at its height).

Certain properties of the hydrazines as MAOI need to be pointed out to place in proper context some of the author's studies of the quaternary nitrogen compounds. The hydrazines and tranylcypromine require preincubation with MAO in order to exert their maximum inhibitory effect, whereas the harmala alkaloids do not require preincubation. The hydrazines and tranylcypromine are irreversible inhibitors once they have had prolonged contact with MAO, manifested by the fact that extensive dialysis does not diminish the extent of inhibition. The harmala alkaloids are reversible, competitive inhibitors. Both of these classes of compounds will raise brain 5-HT and catecholamine levels in various organs (e.g. in brain and heart) of many species, including the rat.[189, 195, 196] Studies of these inhibitors <u>in vitro</u> have invariably been done on particulate MAO because it has never been entirely

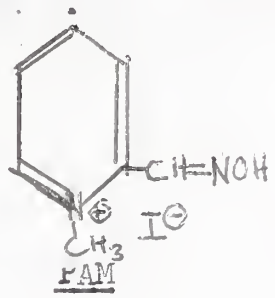
freed from its mitochondrial matrix. In one study, MAO solubilized with the detergent isoctylphenoxypolyethoxyethanol was used.[197] In the presence of this solubilizing agent, MAO activity will not sediment despite centrifugation conditions sufficient to bring down microsomes. (Recently MAO has been obtained from homoginatea subjected to sonic oscillations in a form which did not sediment after centrifugation at 35,000g for 30 minutes without the presence of a detergent).[198]

While investigating new inhibitors of MAO, the author discovered that various compounds which contained a quaternary nitrogen atom, usually in the form of a quaternary pyridinium moiety, were effective MAOI. At first, it seemed that the possibility of this class of compounds being of clinical or even of investigative use, was slight because of the evidence that highly ionic molecules cannot penetrate the blood-brain barrier.[199] However, it seemed likely that a long-chain N-alkyl group would enhance the lipid solubility of the quaternary pyridinium molecule and thus allow it to penetrate the blood-brain barrier, which is known to be most permeable to compounds of high lipid solubility.[200] With this in mind, pyridine aldoxime dodecyliodide (PAD), which has a quaternary pyridinium nucleus with a long chain N-alkyl radicle, was investigated as a possible inhibitor of MAO.



PAD

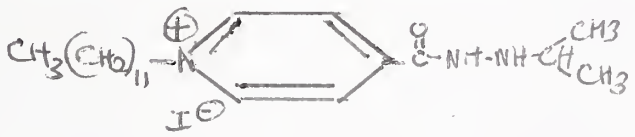
PAD was conceived and synthesized by Wilson and Ginsburg[201] to obtain a more lipid-soluble analog of pyridine aldoxime methiodide (PAM)[202] which was designed and shown by Wilson[203] to be



an extraordinarily effective reactivator of cholinesterase inhibited by organophosphorous compounds such as diisopropylfluorophosphate. Wilson reasoned that because of the N-alkyl radicle PAD would be more likely than PAM to penetrate the blood-brain barrier and to reactivate organophosphorous-inhibited brain cholinesterase. PAM itself has been shown to enter the brain [204] although no similar direct evidence is available that PAD can pass the blood-brain barrier. (The studies presented in this paper strongly suggest that PAD and related compounds gain entrance into rat brain). PAD (in conjunction with atropine) has been shown to be an effective antidote in poisoning, due to organophosphorous cholinesterase inhibitors.[205] In addition to reactivating cholinesterase, PAD can also inhibit cholinesterase, block conduction at the node of Ranvier, increase the permeability of nerve fibers to sodium ions[206] and depolarize resting nerve fibers.[207] Some of these effects have been attributed to the reaction of PAD with the postulated acetylcholine receptor protein along the nerve axon.[206] The work presented here shows that, in addition to these effects on acetylcholine

metabolism and on the proteins associated with it, PAD exerts two important effects on the metabolism of 5-HT: (i) inhibition *in vitro* of MAO, the enzyme that is mainly responsible for the catabolism of 5-HT; and (ii) release of 5-HT from brain and mast cells in which 5-HT may be bound to particulate components. Most of these studies with PAD have been published.[209, 210]

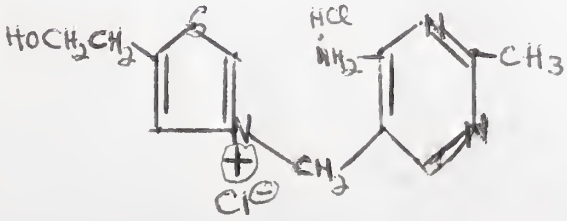
The studies with PAD indicated it would be very interesting to investigate dodecyl iproniazid iodide, the long-chain N-alkyl derivative of iproniazid. This



Dodecyl Iproniazid Iodide

compound would have two functional groups that could inhibit MAO, at least *in vitro*, as well as being able to release 5-HT. Synthesis of this compound was accomplished in the Lederle Laboratories and a small amount was graciously supplied to the author.

Early in the study of the effect of quaternary nitrogen compounds on MAO, the author felt that it might be profitable to investigate the role of thiamine in MAO because thiamine has a quaternary nitrogen in its thiazole moiety.



Thiamine Hydrochloride

Preliminary studies with thiamine - deficient animals revealed brain MAO to be elevated, which could come about if thiamine or a metabolite of it were natural endogenous MAOI. The elevation of brain MAO in thiamine-deficient rats was independently confirmed and published by Gal and Drewes[211] who, whoever did not speculate on any role thiamine might play as a MAOI. A few experiments and an intensive literature search which unearthed a number of relevant findings enabled the author to publish some speculations about the effect of thiamine on MAO.[212] These will be detailed in the Discussion section of this thesis.

MATERIALS AND METHODS

5-HT creatinine sulfate and dl-5-hydroxytryptophan (5-HTP) were obtained from the California Corporation for Biochemical Research. Kynuramine was obtained from the Regis Chemical Company; decamethonium, hexamethonium, PAM, and cetyl pyridinium iodide were purchased from the K & K Chemical Company. Stigmonene bromide was a gift of the Warner-Chilcott Company. Iproniazid phosphate was a gift from Hoffman-LaRoche, Inc. Catron (JB-516 was a gift from Lakeside Laboratories. Harmaline was a gift of Professor Nicholas Giarman of the Yale Department of Pharmacology. Thiamine and thiamine pyrophosphate were purchased from Schwartz Biochemicals, as was diphosphopyridine nucleotide. Succinylcholine, d-tubocurarine, neostigmine and N-methyl nicotinamine were obtained from various commerical sources. PAD was synthesized by the method of Wilson and Ginsburg.[60] Long-chain alkyl pyridinium compounds were synthesized by the method of Knight and Shaw.[213] Dodecyl iproniazid iodide was synthesized by the Lederle Laboratories; the author is very grateful to Dr. Paul Bell, Director of Biochemical Research, Lederle Laboratories, for supplying this compound.

5-HT was determined spectrophotometrically by the method of Udenfriend et al.[101] Numerous experiments were performed to show that PAD does not interfere with the determination of 5-HT by this method. Catecholamines were determined by a spectrofluorometric techniques according to the method

of Bertler et al.[214] MAO activity was assayed spectrophotometrically.[110] All incubations were for 10 minutes within the housing of a Beckman DU spectrophotometer. The results obtained were occasionaly checked by another method, involving the liberation of ammonia.[107,108] 5-HTP decarboxylase was determined by the technique of Gaddum and Giarman.[215] Mitochondria were prepared from rat liver according to the method of Hogeboom,[216] and frozen at -20°C until needed. Each milliliter of the mitochondrial suspension used contained the mitochondria from 1 g of liver (wet weight). MAO from such mitochondrial preparations was "solubilized" by the method of Zeller et al.[217] All compounds were administered to animals intraperitoneally. The rats used were males of approximately 150 -g weight (Sprague-Dawley strain). The mice were DBA/2 males weighing about 20g. The mast cells used were the Dunn-Potter murine mastocytoma, ascites form.[96] PAD was dissolved in a solution containing $NaHCO_3$, 1 mg/ml. In all experiments involving PAD, the control animals were given the $NaHCO_3$ solution.

EXPERIMENTAL RESULTS

(1) Effect of PAD on the MAO activity of homogenates of liver and brain of various species.

Table 1 demonstrates that PAD can inhibit the MAO of both liver and brain of a number of species. Because of the crudeness of these preparations, it is not possible to say whether MAO of brain or liver is more susceptible to inhibition by PAD.

TABLE 1. EFFECT of PAD on the MAO activity of LIVER and BRAIN HOMOGENATES of RAT, RABBIT, GUINEA PIG, and MOUSE

	% Inhibition of MAO			
	Liver		Brain	
Species	10^{-4}M*	10^{-5}M	10^{-4}M	10^{-5}M
Rat	90	25	41	16
Rabbit	69	20	60	20
Guinea Pig	100	20	40	12
Mouse	66	18	45	15

The brains and livers of adult animals killed by decapitation were excised, homogenized in 4 vol of cold distilled water, and filtered through cheesecloth; 0.2 mi of brain homogenate and 0.1 mi of liver homogenate were used for each assay of MAO activity. All assays were done in duplicate with less than 5% variation.

* Concentrations of PAD.

(2) Effect of PAD on MAO activity of washed rat liver mitochondria and comparison of PAD with other inhibitors of MAO.

Table 2 shows that the inhibition of MAO *in vitro*, obtainable with PAD, is substantial. As can be seen in Table 3, PAD is less potent than is JB-516 (Catron, one of the most potent hydrazines used clinically) as an inhibitor of MAO *in vitro*, but is more potent than iproniazid. Table 3 also indicates that a number of long-chain N-alkyl quaternary pyridine compounds can inhibit MAO *in vitro*.

TABLE 2. EFFECT of PAD on MAO activity of Washed RAT LIVER MITOCHONDRIA in vitro

Concentration of PAD	% Inhibition of MAO activity
10^{-4}M	100
5×10^{-5}M	75
10^{-5}M	40
4×10^{-6}M	20

Preincubation of enzyme and PAD was not performed. Four determinations at each concentration of inhibitor were performed with less than 5% variation.

TABLE 3. COMPARISON of PAD and Related Compounds with Phenylisopropyl-hydrazine and iproniazid as MAO inhibitors in vitro

Inhibitor (10^{-5}M)	% Inhibition
JB-516	100
PAD	40
Isonicotinic acid dodecyliodide	40
Dodecyl pyridine iodide	25
Tetradecl pyridine iodide	20
Cetyl pyridine iodide	20
Iproniazid	5

JB-516 and iproniazid were preincubated with the washed rat liver mitochondria for 15 min. before kynuramine was added. Four assays with each inhibitor were performed with less than 5% variation.

(3) Effect of preincubation on the inhibition of rat liver mitochondrial MAO by PAD.

PAD, unlike hydrazine-derived inhibitors of MAO, does not require preincubation to exert its maximal inhibitory action on MAO. Table 4 gives the results of an experiment showing that preincubation of MAO with PAD produced no effect on the inhibition obtained.

TABLE 4. EFFECT of PREINCUBATION on the INHIBITION of MITOCHONDRIA by PAD

Concentration of PAD	MAO activity (ΔO.D./10 min) No preinc.	Preinc.
4×10^{-5}M	0.172	0.170
	0.100	0.100

The substrate, kynuramine, was added to the reaction mixture prior to the addition of MAO, or 30 min. after MAO. In both series, reactions were run in duplicate with and without PAD.

(4) Effect of dialysis on the inhibition of rat liver Mitochondrial MAO by PAD.

The hydrazine inhibitors irreversibly inhibit MAO, while harmaline is a reversible inhibitor.[189] The inhibition due to PAD is not reversible by extensive dialysis, despite the fact that no preincubation is necessary. The results given in Table 5 show that, in an experiment comparing harmaline and PAD, only the harmaline inhibition of MAO was reversible.

TABLE 5. Effect of Dialysis on PAD and Harmaline Inhibition

Mitochondrial MAO

Inhibitor	Δ O.D./10 min/1 No dialysis	ml of mixture Dialysis
	0.130	0.110
PAD (1.5×10^{-3}M)	0.0	0.0
Harmaline (1.5×10^{-3}M)	0.0*	0.60

Of the mitochondrial suspension, 0.4 ml was added to 7.6 ml of a solution of $NaHCO_3$, 1 mg/ml. The same amount of mitochondria also was added to 7.6 ml of the $NaHCO_3$ solution which contained PAD at a concentration of 1.5×10^{3} M. Each mixture was divided in half. One-half was dialyzed for 24 hr. against 3 changes of 4 L of distilled water at 4° C; the other half was left in a test tube at 4°C. After 24 hr. 1-0 ml. of each mixture was test for MAO activity. The same experiment was performed simultaneously with harmaline at the same concentration (1.5×10^{-3} M). These experiments were performed twice with less than 5% variation.

*MAO activity could not be determined because harmaline absorbed too strongly at 360 mu at this concentration; however, harmaline has been reported to inhibit MAO completely at concentrations much lower than this.[189]

(5) Effect of PAD on solubilized MAO.

It might be argued that, because of its detergent-like structure, PAD exerts a nonspecific solubilizing action on MAO. This was tested by observing the effect of PAD on MAO solubilized with isooctylphenoxypolyethoxyethanol. At three different concentrations of PAD, the percentage inhibition of the solubilized enzyme and of that in the mitochondrial suspension were the same (see Table 6) although the total activity of the solubilized enzyme was only one-fifth as great. This is in contrast with the findings of Cotzias *et al.*[197] who noted an increase in enzyme activity after solubilization.

TABLE 6. EFFECT of PAD on "Solubilized" MAO, as compared with the Mitochondrial MAO

Concentration of PAD	% Inhibition of mitochondrial MAO	% Inhibition of solubilized MAO
2.5×10^{-4}	100	100
5.0×10^{-5}	74	74
1.0×10^{-5}	40	40

Cutscum was added to the mitochondrial suspension to make a 5% solution. It was shaken intermittently in the cold for 4 hr and then centrifuged at 100,000 x g for 1 hr. The supernatant fraction was decanted and tested for MAO activity and inhibition by PAD. This was compared with the activity and inhibition obtained with another aliquot of the mitochondrial MAO. All assays were done in duplicate with less than 5% variation.

(6) Ability of PAD to inhibit MAO *in vivo*.

PAD was administered intraperitoneally in various regimens to rats which were then sacrificed at varying times after the last dose. The range of dosages was from 10 to 16 mg/kg for 1-7 days, and the elapsed time after the last administration of PAD was 1-5 hr. Brains and livers were treated as in (1). Little difference could be detected by this method in the activity of the homogenates on MAO of control and treated animals, whereas animals given comparable dosages of the hydrazine inhibitors showed extensive or complete inhibition of MAO.

(7) Effect of PAD on the levels of 5-HT in rat brain.

A sensitive indicator of MAO inhibition, *in vivo*, is an increase in the brain level of 5-HT. It was therefore of interest to determine whether PAD could raise this level. The results, shown in Table 7, indicate that PAD lowered rat brain levels of 5-HT when these were determined 4 hours after the last administration of PAD; 24 hours after a dose that produced extemsive lowering of 5-HT in brain, the level had returned to normal.

TABLE 7. Effect of PAD on 5-HT levels in Brain

PAD (mg/kg)	No. of days administered	Change (% +)
1. 30	1	-15 + 2
2. 15	7	-36 + 4
3. 20	2	-40 + 4
4. 50	2	-64 + 5
5. 65	1	No change 24 hr after injection

Drugs were given intraperitoneally. Each group consisted of 6 male rats. In the first 4 experiments the treated animals and controls were killed by exsanguination 4 hr after PAD and their brains homogenized in 5 ml of 0.1 N HCl. In the fifth experiment the animals were killed 24 hr after administration of PAD.

(8) Effect of PAD on the catecholamine levels of the rat brain.

No effect on rat brain catecholamines was observed when high doses of PAD were given in intraperitoneally for either short or long periods.

(9) Effect of PAD on 5-hydroxytryptophan decarboxylase of rat kidney *in vitro*.

PAD, 10^{-3}M, produced no significant inhibition of rat kidney 5-hydroxytryptophan decarboxylase.

(10) Effect of PAD on 5-HT of mast cells.

The ascites mast cells of the Dunn-Potter mouse mastocytoma contains 5-HT which behaves as though it were bound in granules in pharmacologic experiments.[218] These cells are more suitable for studies on amine release than are the non-nucleated platelets that often are used. Table 8 shows that PAD and other long-chain quaternary pyridinium compounds, but not PAM, released 5-HT from mast cells. JB-516 also caused a similar release.

TABLE 8. Release of 5-HT from Mast Cells

Drug (0.5 mg/ml)	% Release
	20
PAD	100
Tetradecyl pyridine iodide	100
Cetyl pyridine iodide	100
PAM	20
JB-516	60

The mast cells were removed from DBA/2 mice by aspiration of the peritoneal contents, and suspended in Hank's medium.[219] Duplicates were incubated in 20-ml beakers at 37° for 2 hr in a Dubnoff shaker, with and without inhibitors dissolved in Hank's medium. Each beaker contained approximately 10^8 cells. After incubation the cells were centrifuged and the supernatant fractions decanted. The cells were washed with more Hank's medium, centrifuged, and the 2 supernatant fractions combined. The 5-HT content of both the cells and the supernatant fractions were measured spectrophotofluorometrically. There was less than 5% variation in the duplicates.

(11) Effect of PAD and JB-516 on levels of 5-HT in rats after administration of reserpine.

It is conceivable that the release of 5-HT by PAD in the rat brain masked some inhibition of MAO that otherwise would have been detected through increased levels of 5-HT. It was thought that the administration of PAD, after maximal release of 5-HT by reserpine, might then permit the detection of MAO inhibition through the accumulation of newly synthesized 5-HT. Table 9 shows that inhibition of MAO could not be demonstrated *in vivo* even by this method whereas that caused by JB-516 is readily apparent.

TABLE 9. 5-HT Levels after Reserpine followed by either PAD or JB-516

Compound administered	5-HT levels (μg)	Range
Reserpine	0.09	0.08-0.10
Reserpine + PAD	0.09	0.08-0.10
Reserpine + JB-516	0.46	0.43-0.49

Eighteen rats were given 4 mg of reserpine per kg; 6 of these were given 15 mg of PAD per kg. and 6 were given 10 mg of JB-516 per kg. 5 hr after the reserpine. The 5-HT levels at 1 hr after the last injection were determined.

(12) Effect on rats of pretreatment with PAD followed by reserpine, and pretreatment with reserpine followed by PAD.

Pretreatment of rodents with an inhibitor of MAO can reverse the sedative effects of reserpine.[175] Since PAD possesses some of the properties of each of these compounds, it was of interest to observe the performance of PAD in both these roles. As can be seen in Table 10, the behavior of PAD was not typical of an inhibitor of MAO, since it did not reverse the sedative effects of reserpine when given as pretreatment; rather, it delayed them. On the other hand, PAD did not act like reserpine, since it did not produce hyperactivity in rats pretreated with JB-516.

TABLE 10. Effect on Rats of Pretreatment with PAD followed by reserpine, and of pretreatment with JB-516 followed by PAD

Drug administered	Behavioral observations
Reserpine	Sedation in 20 min
PAD followed by reserpine	Sedation in 60 min
JB-516 followed by reserpine	Enhanced alertness and activity
JB-516 followed by PAD	No change from untreated animals
JB-516	No change from untreated animals

Each group consisted of 6 animals; these were kept in individual cages. Observations were continued for 4 hr. All doses (mg/kg) were: reserpine 5; PAD, 50; JB-516, 20. PAD was given for 2 days, the last dose 4 days prior to reserpine or 30 min after JB-516. JB-516 was given 30 min before PAD or reserpine.

(13) Effects of various quaternary ammonium derivatives on MAO *in vitro*.

Of many readily available compounds tested, decamethonium (85% inhibition at 10^{-3}M), hexamethonium (25% at 10^{-3}M), and thiamine (70% at 10^{-2}M) exhibited weak inhibitory actions. Choline, acetylcholine, PAM, diphosphopyridine nucleotide, N-methyl nicotinamide, succinylcholine, d-tubocurarine, stigmonene bromide, and neostigmine were inactive. Several short-chain N-alkyl derivatives of heterocyclic nitrogen compounds were also good inhibitors *in vitro*.

(14) *In vivo* and *in Vitro* Studies with Thiamine and MAO.

As mentioned previously, studies by the author and Gal and Drewes[211] demonstrated that in thiamine-deficient rats the MAO-activity of brain and intestine is increased. However, rats given thiamine, 70mg/kg subcutaneously, and sacrificed after 2 hours, when thiamine pyrophosphate levels in liver are elevated,[220] showed no inhibition of the MAO-activity of liver, brain, and intestine, as compared with untreated controls. Studies *in vitro* showed that thiamine at a concentration of 10^{-2}M inhibits the MAO of rat liver mitochondria 70%, whereas thiamine pyrophosphate is slightly less effective.

(15) Effect of Dodecyl Iproniazid Iodide on MAO *in vitro*.

Table 11 demonstrates that dodecyl iproniazid iodide is an effective inhibitor of rat liver mitochondrial MAO without any preincubation, but that is an appreciably more effective inhibitor after preincubation.

Table 11. Effect of Dodecyl Iproniazid Iodide on MAO Activity of Washed Rat Liver Mitochondria With and Without Preincubation

Concentration of Dodecyl Iproniazid Iodide	% Inhibition of MAO No Preinc.	Preinc.
1×10^{-4}M	45%	95%

The substrate, kynuramine, was added to the reaction mixture which contained dodecyl iproniazid iodide prior to the addition of mitochondrial MAO, or 30 minutes after the MAO. In both series, reactions were run in duplicate with and without dodecyl iproniazid iodide.

(16) Ability of Dodecyl Iproniazid Iodide to Inhibit MAO *in vitro*.

Dodecyl iproniazid iodide, 20 mg/kg, was given introperitoneally to rats which were sacrificed four hours later. Liver specimens were treated as in (1). Dodecyl iproniazid iodide produced a 70% inhibition of rat liver MAO under these conditions.

(17) Effect of Dodecyl Iproniazid Iodide on the Levels of 5-HT in Rat Brain.

Dodecyl iproniazid iodide, 20 mg/kg, was given intraperitoneally to 6 rats which were sacrificed four hours later. Brains were excised and treated as in (7). Dodecyl iproniazid iodide increase 5-HT levels by 50% under these conditions.

DISCUSSION

PAD as an Inhibitor of Monoamine Oxidase

These studies demonstrate that the long-chain N-alkyl quaternary pyridinium compounds have a unique series of effects on brain amines and on MAO. PAD was studied extensively as a prototype of this class, but some of the experiments performed demonstrate that these properties are probably possessed by most or all of the whole class of compounds.

PAD ranks among the most potent MAO inhibitors *in vitro* when related to published comparative data from studies with iproniazid,[47-53] and the author's studies with PAD, iproniazid, and other MAOI. PAD irreversibly inhibited MAO *in vitro*, without preincubation. This sets it apart from the "hydrazines," which required preincubation, and from the harmala alkaloids, which are reversible inhibitors. Dodecyl iproniazid iodide, which was designed as a combination of a hydrazine MAOI and a long-chain N-alkyl pyridinium compound, was found to be an effective inhibitor without preincubation, but to be an even more effective inhibitor after preincubation. This could be due to two separate modes of MAO inhibition: that attributable to the N-alkyl pyridinium moiety, not requiring any preincubation and accounting for most of the 45% inhibition due to the hydrazine grouping requiring preincubation and accounting for the additional 50% inhibition noted after 30 minutes preincubation. As mentioned above, the potency

of PAD as an *in vitro* inhibitor is considerable, and it is not unreasonable to predict that more potent inhibitors of MAO, containing a quaternary pyridinium nitrogen, can be synthesized.

Every attempt to demonstrate inhibition of MAO *in vivo* with these compounds was unsuccessful: (i) There was no decrease in the MAO activity of liver and brain homogenates of PAD-treated animals; (ii) 5-HT levels in brain *decreased* rather than increased after treatment with PAD; (iii) PAD produced no increase in the 5-HT levels of rats pretreated with reserpine to release nearly all of the 5-HT initially present, thus eliminating the possibility that release of 5-HT by PAD was obscuring a simultaneous accumulation due to inhibition of MAO. Rats given JB-516, a potent MAOI, readily accumulated 5-HT under the same conditions indicating that after the initial release by reserpine and degredation by MAO, newly formed 5-HT would accumulate, though its binding might be impaired by the reserpine. In the PAD-treated animals no accumulation was noted, probably because MAO was not inhibited. (iv) Finally, there was no reversal of the reserpine-induced depression in rats pretreated with PAD.

There are several questions to be considered in seeking an explanation for the absence of *in vivo*-inhibition of MAO. Can PAD penetrate the rat blood-brain barrier and reach the cells of the central nervous system? Can PAD

enter the various types of cells of the rat liver? If penetration does occur, was the amount of PAD administered adequate to produce detectable inhibition?

The evidence for the ability of PAD and other quaternary pyridiniums to penetrate the rat blood-brain barrier is the decrease in the 5-HT levels which PAD produces, and the increase in brain levels of 5-HT which dodecyl iproniazid iodide produces. The release of 5-HT from mast cells *in vitro* by PAD supports the hypothesis that the release is mediated directly by this agent rather than in some indirect manner. There is the possibility that the releasing effect is produced by less PAD than is required for the inhibitory effect on MAO, so that sufficient PAD enters the brain to produce the former but not the latter effect. The exclusion of PAD from the rat liver is another possibility that cannot yet be dismissed, although it is not a very attractive one. The demonstration of the inhibition of liver MAO by dodecyl iproniazid iodide makes this very unlikely.

The question of adequate dosage is less troublesome, as can be shown by comparing PAD with iproniazid. After rats were given a single dose of iproniazid of 5×10^{-5} moles/kg body weight, MAO activity determined subsequently *in vitro* did not return to normal for 5 days.[221] In the most vigorous attempt to demonstrate MAO inhibition by PAD, 1.4×10^{-4} moles/kg of PAD was given to each of the six rats daily for

seven days, and no inhibition of MAO of brain or liver was detected in any rat, despite the evidence presented herein that PAD, like iproniazid, is an irreservible inhibitor, and that the *in vitro*-assay used in these experiments[110] is more sensitive than is the one formerly in use.[221] Another possible explanation is that PAD is rapidly detoxified *in vivo* but not *in vitro* by the mitochondria. Thus, *in vivo*, PAD which reached amine-containing granules before reaching mitochondria could exert its releasing action, whereas the mitochondrial detlxification would prevent PAD from inhibiting MAO, a mitochondrial enzyme. If so, analogs of PAD that are not rapidly inactivated *in vivo* might be synthesized, and a useful MAOI might result. However, recent studies of the metabolism of PAM in man[222] and in lower animals[223] revealed that the drug is excreted largely unchanged, although small amounts of a number of other metabolites were noted. Whether the same holds true for PAD remains to be established.

Perhaps the best explanation for the difference found *in vitro* and *in vivo* is the recent work by Aebi.[224] Aebi examined mitochondrial MAO in various states of preservation of the structure of mitochondria and showed that the enzyme behaved differently under each set of circumstances. The activity of MAOI was also influenced by the morphologic state of the mitochondria. The effectiveness of the MAOI studied, varied with the intactness of the mitochondria

leading Aebi to conclude that it was very difficult to predict what results would be obtained in the intact state (*in vivo*) from studies on mitochondria that were to some extent disrupted (*in vitro*). Pletscher and Gey[225] have noted many other compounds which inhibit MAO *in vitro* but not *in vivo*. Werke *et al.*[226] have recently reported that N-benzyl -N' - isopropylhydrazine inhibits MAO *in vivo* but not *in vitro* and speculate that many effective inhibitors have probably been missed because most screening methods for new MAOI are of an *in vitro* nature.

The weak ability of hexamethonium to inhibit MAO is of some interest in the light of the recent demonstration that inhibitors of MAO can produce ganglionic blockade.[176] This is not to suggest that the effects of hexamethonium are due to MAO inhibition; rather, it is meant to point up the reciprocity between these two activities that might lead to an exploration of other similarities. It is interesting, moreover, that hexamethonium potentiated the effects of 5-HT in some experiments with the isolated guinea pig ileum.[227] Could this have been due to MAO inhibition?

In the same light, the actions of PAD on both acetylcholinesterase and acetylcholine receptors, and on MAO may be reiterated. It would be on interest to determine whether the influences of PAD on the release of 5-HT and on the activity of MAO are of any importance for understanding the observations of Dettbarn and Wilson already alluded to.[206,207]

It is worthy of note that chlorpromazine[228] and tolazoline[229] also inhibit both MAO and cholinesterase.

PAD and Amine Release

In experiments to determine whether PAD could increase 5-HT levels as would be expected of a MAOI, it was noted that instead of increasing 5-HT levels, PAD actually produced a decrease in the amount of the amine. The extent of lowering was found to be dependent upon the dosage of PAD. That 5-HT release accounted for the decrease in 5-HT levels noted in Table 7 is indicated by the inability of PAD to inhibit 5-HTP decarboxylase and the ability of PAD to produce similar changes in the 5-HT levels of mast cells (Table 8).

In the experiments with mast cells, the 5-HT which sedimented with the cells and which was present in the supernatant fluid was determined with and without treatment of the cells with PAD, other quaternary pyridinium ions, etc. All the 5-HT was recovered in either the supernatant fluid or the cell mass. The quaternary pyridiniums, including PAD, effected a transfer of the 5-HT from the cells to the supernatant fluid. This strongly suggests release of the amine.

The amine-releasing action of PAD was an unexpected finding and added a new dimension of interest to the study of this compound. Firstly, because the mechanism of many of the clinically and experimentally valuable actions of

the rauwolfia alkaloids, particularly reserpine has been thought to be related to amine-release.[230-232] It is likely that study of other amine-releasing agents can help illuminate this matter. PAD is particularly useful in this regard, because it is a matter of heated dispute whether it is through 5-HT or catecholamine depletion that reserpine brings about its actions. This problem has been approached by using drugs such as tetrabenazine,[233] O-methyl-meta-tyrosine,[234] conditions such as reserpine plus hypothermia,[235] or a diet deficient in 5-hydroxytryptophan[236] to deplete selectively either the catecholamines or 5-HT and to determine whether any correlation between behavior and cerebral change in one or the other amines could be obtained. At this point, there is good evidence in both camps, (perhaps an indication that neither amine is involved). Nevertheless, the studies reported here indicate that PAD can be of use in this problem. No other agent has been reported to the author's knowledge, which depletes bring 5-HT but not the catecholamines, although it has many other effects, as previously noted. This selective action of PAD could be exploited to elucidate the physiologic import of 5-HT depletion. Qualitatively, animals treated with doses of PAD which depleted 65% of their 5-HT seemed no less active and alert than controls who had been treated identically, except for not receiving PAD. The studies detailed in Table 10 indicated that PAD pretreatment delayed, not hastened the onset of sedation

in rats given small doses of reserpine. Also rats pretreated with phenylisopropylhydrazine (JB-516), an effective MAOI, were alerted and activated by reserpine, whereas a high dose of PAD had no effect on animals pretreated with phenylisopropylhydrazine. These differences in the effects of PAD and reserpine, although admittedly subjective and in need of quantitative verification, indicate that if amine-release is the means by which reserpine produces behavioral effects, it is mediated via change in catecholamines, rather than 5-HT. (It is of course possible, that it is another amine, or no amine at all, through which reserpine operates). The second reason for being interested in the amine-depleting action of PAD is that it is the only compound which has been shown to both release brain amines and inhibit MAO, Tranylcypromine, one of the MAOI mentioned previously, has been shown to release norepinephrine from the hearts of both rats and cats,[237] but it has not been observed to release brain amines. Iproniazid can diminish the monoa mines in the cat hypothalamus but this was thought to be a non-specific effect.[238] It is of interest that PAD's effect on 5-HT binding is not long-lasting. Twenty-four hours after a large dose of PAD, 5-HT levels return to normal.

If a quaternary pyridinium molecule were synthesized which could inhibit brain MAO in vivo as well as release brain 5-HT, it might turn out to have unique behavioral and possibly therapeutic effects since it is by no means

certain that it is through catecholamine release rather than through 5-HT release by which reserpine acts, or that 5-HT depletion has no behavioral effect. Studies on the treatment of depression in man with a combination of reserpine and iproniazid, i.e., with an amine releaser and a MAOI, have indicated that this combination has particular value.[239]

Dodecyl Iproniazid Iodide

The studies with dodecyl iproniazid iodide showed that it possessed the properties that were predicted by the author. The inhibition of MAO *in vitro* without preincubation was probably due to the N-alkyl group bonded to the pyridine nitrogen atom, whereas the supplemental inhibitory potency noted after preincubation was probably due to the hydrazine moiety. Studies *in vivo* demonstrated that it is a potent inhibitor of liver MAO (70% inhibition at 20 mg/kg) and that it raises brain 5-HT levels. This implies that it inhibits brain MAO also, or prevents the release of brain 5-HT. It could also release brain 5-HT despite the 50% elevation of 5-HT over control values because the simultaneous inhibition of MAO would prevent its destruction so it might accumulate in its "released" state. There were many interesting experiments to do with this compound, but unfortunately only a very small amount was available to the author and the method of synthesis was not available.

The effects of PAD and dodecyl iproniazid iodide on rat brain imply that both these agents can pass the blood-brain barrier and provide additional evidence for Wilson's idea[201] that long-chain N-alkyl radicals can be used to enhance the ability of highly ionized molecules to penetrate this barrier.

The Effect of Thiamine on Monoamine Oxidase

In a recent publication,[211] Gal and Drewes demonstrated that in thiamine-deficient rats, the MAO-activity of the brain and intestine is increased, as compared to the activity of rats fed *ad libitum* with a normal laboratory diet. These workers suggested that the increase might be related to the stress theory of Selye. However, it is proposed by the author, that, *in vivo*, thiamine, which has a quaternary nitrogen in its thiazole moiety, is either itself an inhibitor of MAO, or more likely, is metabolized via a pathway which produces another quaternary nitrogen compound which is an inhibitor of MAO. Thus, the increased MAO-activity in the thiamine-deficient animals might be accounted for by a diminution in the amount of this thiamine-like inhibitor. The MAO-inhibition caused by this thiamine-like compound also might explain the hypotension and ganglionic blockade which thiamine produces in man and experimental animals.[240,241] Gertner has shown that MAOI block ganglionic transmission in cats and dogs,[176] while hypotension in man has been noted with the administration of MAOI.[180]

Experiments presented here show that thiamine and thiamine pyrophosphate *in vitro* are weak inhibitors of MAO: at 10^{-2}M thiamine inhibits the MAO of rat liver mitochondria 70%, a result of little significance with respect to the proposed mechanism *in vivo*. Furthermore, rats given 70 mg of thiamine per kg subcutaneously, and sacrificed after two hours, when thiamine pyrophosphate levels in liver are elevated,[220] showed no inhibition of the MAO-activity of liver, brain and intestine as compared with untreated controls. However, this does not constitute a refutation of the proposed theory, if the rate of formation of the postulated thiamine metabolite proceeds maximally with the amount of thiamine provided by a normal diet.

Substantial indirect evidence can be provided for the theory. Although in higher animals most of the thiamine which is absorbed is excreted unchanged, the fate of the thiamine which is degraded, is largely unknown.[242] Molluscs, carp, and some other lower species possess an enzyme, thiaminase,[243] which catalyses the reaction of thiamine with a variety of basic compounds with loss of the 4-methyl-5-hydroxyethylthiazole portion, and the formation of other quaternary compounds derived from the pyrimidine portion.[243] No similar enzyme has been found in mammalian tissue, but only small amounts may be present. Such a reaction could produce a potent quaternary inhibitor of MAO. It is significant that Minz[244] and von Muralt,[245,246] using labeled thiamine, has shown that, on excitation of a peripheral nerve,

metabolites of thiamine appear, which are as yet unidentified. Other studies have shown that excitation of the vagal nerve can release thiamine into the heart.[247-250] Four quaternary ammonium ions, one of them a quaternary pyridinium molecule have been found in the giant nerve fibers of the squid.[251] One of these might be the postulated inhibitor; if so, this would provide a mechanism for preventing the oxidation of any neurohumor which is a substrate for MAO.

The hypotension and ganglionic blockade produced by thiamine and by known inhibitors of MAO is suggestive that these compounds are related. This action of thiamine is unrelated to its properties as coenzyme.[240] This blockade is also different in kind from that produced by hexamethonium,[241] as is that caused by the inhibitors of MAO.[176]

A final argument which may be offered is that in thiamine-deficiency state in man (beri-beri), the initial symptoms are emotional - depression, apathy, and disinterest,[252,253] a syndrome a physician might now treat with an inhibitor of MAO, which indeed would be only replacement therapy, if thiamine administration to thiamine-deficient organisms does in fact produce an MAO inhibitor.

It is interesting that thiamine-deficient rats also show increased cholinesterase activity[116] and diamine oxidase activity,[117] while thiamine is also a weak inhibitor of these enzymes, <u>in vitro</u>. It has been proposed

that thiamine inhibits both of these enzymes *in vivo*.[254,255] Thus, there is both precedent for the theory presented and a wider scope for a mechanism which would delay the catabolism of neurohumors such as acetylcholine, histamine, serotonin, and the catecholamines.

SUMMARY OF PART TWO

1. Pyridine aldoxime dodecyliodide (PAD) was found to inhibit the monoamine oxidase (MAO) of homogenates of rat, rabbit, guinea pig, and mouse brain and liver *in vitro*. This is the first demonstration that a quaternary nitrogen compound can inhibit MAO. Numerous other such compounds were also found to inhibit MAO *in vitro*.

2. PAD is a more powerful MAO inhibitor *in vitro* than iproniazid which at one time was widely used in the treatment of depression.

3. No preincubation of enzyme and inhibitor is necessary and the inhibition is irreversible. This sets it apart from other MAO inhibitors.

4. PAD is also an effective inhibitor of "solubilized" MAO.

5. PAD is not an effective MAO inhibitor *in vivo*. Possible explanations for this discrepency between *in vivo* and in vitro results are discussed.

6. PAD, like reserpine, was found to lower the 5-hydroxytryptamine (5-HT) levels in rat brain, but has no effect on the catecholamine levels. It also releases 5-HT from the Dunn-Potter mouse mastocytoma ascites cells.

7. PAD does not inhibit rat kidney 5-hydroxytryptophane decarboxylase *in vitro*.

8. These findings suggest that PAD exerts a selective releasing action on bound 5-HT. It is the only compound reported which selectively depletes 5-HT but not the catecholamines.

9. PAD treatment produced no accumulation of rat brain 5-HT after initial depletion of 5-HT with reserpine whereas 5-HT did accumulate after treatment with a hydrazine type MAO inhibitor under the same conditions. This is a more sensitive indication that no MAO inhibition occurs *in vivo*.

10. Animals given sufficient PAD to deplete their 5-HT stores do not have the appearance of animals treated with reserpine. In addition, animals pretreated with phenylisopropylhydrazine (PIH), and inhibitor of MAO, and then given PAD do not have the appearance of animals pretreated with PIH and then given reserpine.

11. Decamethonium, hexamethonium and thiamine are also weak inhibitors of MAO *in vitro*.

12. The reported increase in brain MAO activity of thiamine-deficient rats is interpreted as an indication that thiamine or probably one of its metabolites is an MAO inhibitor *in vivo*. Relevant data from the literature is offered in support of this idea.

13. Dodecyliproniazid iodide was conceived of as a molecule combining the MAO inhibitory properties of a hydrazine and the 5-HT releasing properties of a quaternary nitrogen compound. It was found to inhibit MAO *in vitro* with both the hydrazine moiety and the quaternary nitrogen moiety. It was also found to release rat brain 5-HT *in vivo*. This is the first compound to both inhibit MAO *in vivo* and release brain 5-HT and might have unique psychopharmacologic properties.

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